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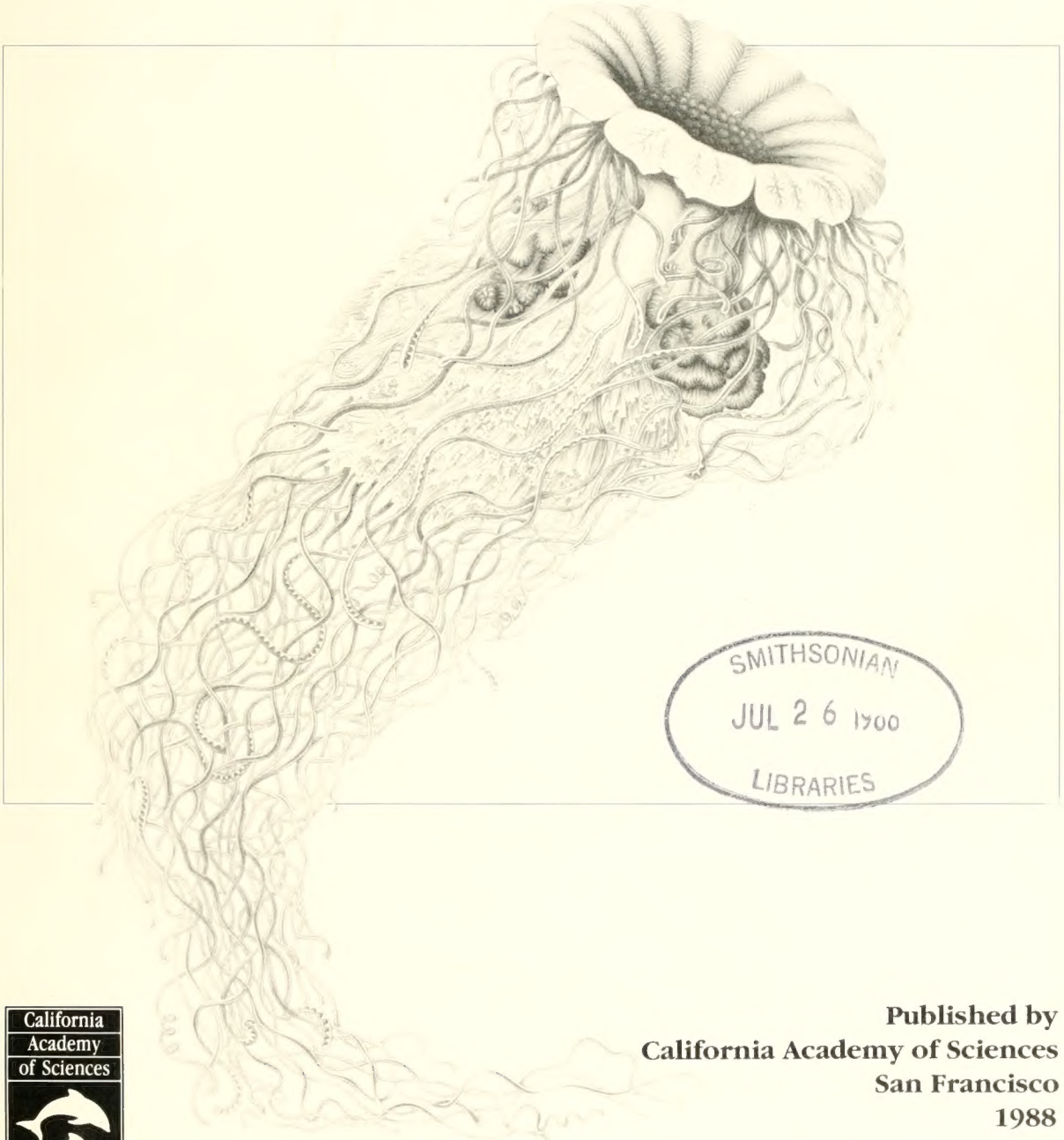
BIOMEDICAL IMPORTANCE OF MARINE ORGANISMS

Edited by Daphne G. Fautin



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Cover Illustration: This jellyfish—a member of the order Semaeostomeae—was rendered by Ernst Heinrich Haeckel (1834–1919), and published in his 1904 book *Kunstformen der Natur* (*Art Forms in Nature*). Haeckel was a specialist in "lower" organisms, many of which, like this jellyfish, exhibit lovely symmetry. However, he was a general zoologist, having founded the Phyletisches Museum in Jena, and originated the "tree of life" diagram, perhaps his most famous zoological rendering. Thus, for several reasons, this is an appropriate symbol for the symposium Biomedical Importance of Marine Organisms.

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INTRODUCTION

The multifaceted field known as marine pharmacology has recently experienced a rapid expansion in pure and applied results, which has led to a resurgence of funding. Whole families of chemical compounds rare or unknown in the terrestrial realm are being characterized. Model systems involving marine organisms have been developed that aid in understanding many physiological processes. Compounds of marine origin are finding increasing use as highly specific probes for investigation of cellular function and structure. The first drugs developed from marine organisms are now in clinical trials, and the National Cancer Institute has launched a new program for collecting and screening the sources of possible therapeutic agents.

At this watershed, I invited 22 speakers, each selected to represent a segment of concern along a broad spectrum of the discipline, to present retrospective and prospective views of those facets, providing an historical context to current trajectories. The purpose of the Third Biennial Symposium of the California Academy of Sciences, held 30 April–2 May 1987, in San Francisco, was to gather together speakers not only from different fields within marine pharmacology, but from government, industry, and academe as well. My explicit intention was to cross-cut conventional boundaries that often prevent people with similar interests from learning about one another's work; this was a forum for the exchange of ideas among colleagues who have few formal opportunities to meet. The symposium was not the place to communicate late-breaking discoveries, nor for minute technical details in conventional research format where peers and competitors are addressed in a language few others might understand—there are plenty of those already. Rather, I sought overviews of topics, with emphasis on implicit or explicit connections between fields and among speakers.

The focus of the meeting is reflected in its title, and that of this resultant volume, "Biomedical Importance of Marine Organisms." As a marine biologist, my primary interest in marine pharmacology is the organisms that are the source of extracts, or that provide models, or that are used as evaluation systems. For other scientists, the organisms may be of secondary or even tertiary concern, but sight of them should never be lost, I believe. It is the plants and animals—as objects and subjects of research, or the sources of parts and molecules—that all participants in the symposium, whether systematists, biochemists, pharmacologists, or physicians, have in common.

This perspective also explains why holding such a meeting is appropriate to a natural history museum. Among the conditions imposed by NCI on contractors in its new program are those dealing with documenting the provenance of the organisms in question, obtaining accurate identifications of them, and deposition of documentary samples in appropriate archival facilities. Many curators at natural history museums, including the California Academy of Sciences, are frequently called upon to identify specimens for physiologists, chemists, and pharmacologists. Thus, the first step in the process of "doing" marine pharmacology involves systematics. The importance of systematics in the ultimate success of the entire process cannot be overestimated, and holding this symposium in a natural history museum is a graphic way to make that point.

The symposium topics are organized along a time-line, in the order with which they are dealt in the actual practice of marine pharmacology. From my perspective, that begins and ends with organismal biology, while chemistry, pharmacology/physiology, and pharmaceutical science are sandwiched between. First, organisms are collected and identified. In the case of extracts for probes or therapeutic use, successive phases of screening and purification come next, with chemical characterization and, possibly, synthesis and drug trials following. Where organs or organisms are of interest as models or test systems, isolations, preparations, and techniques must be developed. For completeness, the focus should eventually return to marine organisms and to the function of the molecule or structure in the plant or animal from which it was derived.

Practitioners of marine pharmacology who deal in organs or organisms seem frequently and explicitly to recognize that molecules and structures represent strategies for dealing with the problems of survival common to all living beings, and that they usually reflect evolutionary relationships. For example, both vertebrates and molluscs evolved sophisticated nervous systems, with image-forming eyes and nerves capable of rapid impulse transmission. Comparative physiological and anatomical research revealed these similarities to be analogous rather than homologous—the animals accomplish similar ends from totally distinct beginnings: nervous transmission is accelerated by myelin sheaths around vertebrate nerves, and by increased diameter in molluscan nerves. Neurophysiologists took advantage of molluscan nerve size, as described by Toshio Narahashi in this volume, to unravel the mechanism of impulse transmission, which, despite the differences in structure, turned out to be the same in vertebrates and molluscs. Therefore, much of what was learned from squid giant axons was directly applicable to vertebrate nervous systems.

Even when direct application is impossible, understanding alternative evolutionary pathways through comparative study of structure and function almost invariably casts light on the system of interest. One of the most intriguing—and ignored—issues to me, an evolutionary biologist, is whether physiologically active compounds act similarly in the organisms producing them and in experimental systems. Biochemists, pharmacologists, and the like can sometimes lose sight of the obvious—compounds they study were not put in marine invertebrates to cure mouse Ehrlich ascites tumor or to puzzle chemists. I appeal for structural and functional studies on extracts from marine organisms to be brought full circle, to address explicitly questions of evolutionary relationships. The body of information already extant can probably shed important light on issues of analogy and homology. This symposium can only hint at the value of two-way information exchange between all possible combinations of people working in this multifarious field, and the gains to be made from keeping marine organisms squarely in the foreground.

Following the time line, the two lead-off papers, by Judy Winston and Shirley Pomponi, address issues of collection and identification of organisms—current techniques and suggestions for their improvement, importance of documentation and accuracy, and what options and resources exist. For those in marine pharmacology to whom finding novel compounds of potential physiological/pharmaceutical importance is paramount, Ken Rinehart explains the philosophy and practice of screening. Selectivity and economy in collecting efforts are enhanced when organisms likely to be of interest can be predicted. Valerie Paul

and John Faulkner do this by studying plants and animals that are members of certain taxonomic groups, or that share particular ecologies—both types of leads are potentially fruitful. An alternative strategy in quest of marine pharmaceuticals—traditional human usage—is presented by Paul Scheuer, who finds that in some instances there seem to be physiological grounds for the practices and beliefs, but that other substances appear to have exclusively magical value.

After promising, or even merely interesting, chemicals are uncovered, they must be characterized. Atoms appear to be put together differently in the sea than on land, as discussed comprehensively by Chris Ireland. Koji Nakanishi provides several instructive examples of the value—and difficulty—of establishing the function of compounds found to be physiologically active in the organisms producing them. Recent technological innovations have made peptide chain compounds accessible to certain kinds of pharmacological studies, according to Bill Kem, who finds remarkable diversity in toxins. The phylogenetic relationships of animals, as evidenced by chemistry of their peptides, is investigated by Mike Greenberg.

Marine organisms are of biomedical importance for reasons other than simply the compounds that might be extracted from them. They have provided model systems to investigate fundamental physiological processes at the molecular level. Perhaps foremost among the beneficiaries of this approach has been neurophysiology. Toshio Narahashi provides several examples with both pure and applied implications. Toxins, which typically demonstrate considerable molecular specificity, have proven to be marvelous probes, in ways and for reasons explained by Palmer Taylor and Doju Yoshikami (who did not submit a written version of his talk for publication), as well as by Bill Kem. The elegant preparation of crustacean antennae by Bill Carr's group for study of chemoreception is a classic example of a marine organism model system.

Manoalide, a sponge natural product, is not only allowing new approaches to the investigation of inflammation, as Larry Wheeler explains, but is a potential anti-inflammatory drug, according to Alex Mayer and Bob Jacobs. Other pharmaceuticals in various stages of development, including antimicrobials, were described by Tom Matthews (who did not contribute to the written record of the symposium).

What if a wonder drug—or even just a moderately good one—is found as a result of marine pharmacognostical research? How can it be obtained in quantity? Several speakers alluded to the unpredictable nature of many compounds of interest: some are present only in certain portions (geographical or bathymetric) of a species's range, others occur seasonally or erratically, whereas some are ephemeral once the organism has been collected. For those reasons, as well as concerns about conservation, harvest is unlikely to provide the quantities needed. Chemical synthesis is an unlikely commercial source, concluded Jon Clardy, in his exclusively oral presentation, since few laboratory syntheses can be translated successfully into large-scale, economical production. Dick Moore's examination of the promises and problems of mass culture were far more optimistic. And, on the cutting edge of technology, Bill Radany described (at the meeting but not in this volume) genetic engineering of a polypeptide from the sea anemone *Anthopleura*.

Prospects for continued support of such research from U.S. governmental sources were reviewed by Matt Suffness and Janice Thompson. In describing long-range objectives of the marine natural products program of NCI, which include study of the natural functions of compounds discovered to be of pharmaceutical interest, they also returned the focus squarely to the organisms themselves.

This stimulating symposium, and its resultant volume, are due in large part to the continuing support and enthusiasm of Executive Director Frank Talbot, who has made the California Academy of Science's biennial symposia a reality. Kathryn Hecht of the Development Office helped raise funds to pay for the many obvious and not so obvious costs. Mary Ford, in the Academy's Exhibits Department, designed the graphics, with a nod to Ernst Haeckel. The Special Events and Travel office, under Sandy Lelich, with the able assistance of Nancy Fuller, coordinated travel and a-v equipment for the speakers, and registered members of the audience. Jim Runner was projectionist. Receptions were orchestrated by Deidre Kernan, of Special Events. Editorial Assistant Chris Cunningham was invaluable in the production of this proceedings volume. But, as usual at the Academy, the paid staff would not have sufficed. Cecelia Beam and Diane Butler of Volunteer Services mobilized a veritable army that assembled hand-outs, dealt with registrations, posted fliers, etc. The symposium would never have become a reality without one of their number, Raine Warner, who assisted me in nearly every aspect of planning and execution.

A major grant (1 R13 CA44702-1) from the National Institutes of Health—especially NCI—made this symposium possible. Additional much-needed and deeply appreciated financial assistance came from Allergan Pharmaceuticals, Inc., Bristol-Meyers Company, Ciba-Geigy Corporation, Herbert Laboratories, Rohm and Haas Company, SeaPharm, Inc., and Syntex Corporation.

Finally, the scientific program was shaped with the advice of an organizing committee: Bill Fenical, Bob Jacobs, and Bill Kem. The latter Bill was also very helpful in producing this volume. And, of course, the ultimate success of the symposium rested with all of the contributors, to whom I express my utmost gratitude.

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The Systematists' Perspective

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INTRODUCTION

> Natural History is far too much a science of dead things; a necrology. It is mainly conversant with dry skins furred or feathered, blackened, shriveled, and hay-stuffed; with objects, some admirably beautiful, some hideously ugly, impaled on pins, and arranged in rows in cork drawers; with uncouth forms, disgusting to sight and smell, bleached and shrunken, suspended by threads and immersed in spirit (in defiance of the aphorism, that "he who is born to be hanged will never be drowned)" in glass bottles. These distorted things are described; their scales, plates, feathers counted; their forms copied, all shrivelled and stiffened as they are; their colors, changed or modified by death or partial decay, carefully set down; their limbs, members and organs measured, and the results recorded in thousandths of an inch; two names are given to every one; the whole is enveloped in a mystic cloud of Graeco-Latino-English phraseology (often barbaric enough);—and this is Natural History.

This quotation from Philip Henry Gosse's preface to *A Naturalist's Sojourn in Jamaica* was published in 1851. It could still serve as what many laymen would consider a definition of systematics. Gosse goes on to say that "careful and minute descriptions, accurate measurements and distinctive names are absolutely indispensible to science, but they must not be confused with science itself." This shows another prejudice with which systematists have been contending for over a hundred years—that what they do is useful, but that it is not science. Even other scientists may share this stereotypical view of systematists as bearded old men sitting in rooms full of tiny skulls or seashells, mindlessly classifying away. Many scientists see systematics as a service occupation, and believe that the primary function of a systematist is to identify specimens for others—a view that can lead to confusion and anger when the systematist refuses to cooperate.

Systematists themselves believe that what they do is not only science, but that it provides one of the fundamental perspectives of biology. Their work is not just a cataloging of dead things. It involves the integration of many fields of biology: ecology, functional morphology, behavior, biochemistry, molecular biology, genetics, etc., in order to determine relationships and account for diversity in nature.

The current plight of systematics in general (the problems of rapidly growing collections, inadequate financial resources, nonuniform standards for documentation and preservation, and shortages of trained personnel, modern equipment, and data processing systems) has been thoroughly discussed in several reports (Steere et al. 1971; Irwin et al. 1973; Lee et al. 1978; Stuessy and Thompson 1981; Edwards et al. 1985; Scudder 1987). For the purposes of this presentation I take a more specialized approach to attempt to determine the state of knowledge of the taxonomy of some important marine plants and animals in natural products chemistry and biomedical research: algae, sponges, coelenterates, nemerteans, bryozoans ascidians, nudibranch molluscs, and echinoderms. Such information is not available in the literature except in a very general way. For example, it has been estimated (Goto 1982) that more than 90% of marine invertebrate species are still undescribed.

Therefore, I went directly to the specialists themselves. I interviewed (by telephone or questionnaire) a number of systematists working on these groups. I asked them for their estimates of the diversity of their group in various geographical areas, the numbers of systematists actively working on their group, and the present state of knowledge of the taxonomy of their group. I asked them to tell me the chief difficulties in identifying the organisms in their field of study. I questioned them on their professional priorities and policies regarding the identification of specimens for others. I hope that the results of this survey will lead to a better appreciation by other scientists of the difficulties faced by systematists working on these groups (and others), and may help to explain why what appears to them to be a simple request for the name of an organism might be difficult or impossible to provide.

WHAT SYSTEMATISTS DO

Biological taxonomy is simply "the theory and practice of classifying organisms" (Mayr 1969), while systematics has been defined by Simpson (1961) as "the scientific study of the kinds and diversity of organisms and of any and all relationships among them."

All the systematists I interviewed agree that research is their first priority, but claim that people outside systematics do not always understand the variety of studies that this "testing of phylogenetic hypotheses" covers. What most people think systematists do is what is called the "alpha level" of taxonomy, describing new species, compiling checklists and catalogs, and making studies of the fauna of a particular area. In groups with many as yet undescribed taxa, such projects can take up much of a systematist's time. However, it is not considered the most creative or sophisticated aspect of the field, and such studies, while publishable and often essential to others, are seldom considered for funding by granting agencies. Most systematists probably spend a greater amount of time on "beta taxonomy," carrying out revisionary studies in order to provide a more phylogenetically accurate classification of a group. Others may work at the level of "gamma taxonomy," pursuing the bases of intraspecific variation via population genetics, biochemical genetics, etc., or reordering higher taxa in an attempt to understand their evolutionary history. Eventually, this research, like that of other scientists, results in papers, ranging from short checklists to enormous monographs, published in the scientific literature. It is on such publications that systematists are judged by their peers and by the administrators of the institutions for which they work.

Other duties of systematists vary. Among those who work in museums the second highest priority is generally given to the maintenance and improvement of collections. Those who are employed by museums with collections management personnel may do little curation themselves; others must do all their own sorting, accessioning, and cataloging. Few of the systematists interviewed were interested only in increasing collections rele-

TABLE 1. DIVERSITY OF MARINE ORGANISMS IMPORTANT IN BIOMEDICAL RESEARCH

Group	No. of species	No. of active systematists
Algae	30,000	40
Sponges	10,000	16
Coelenterates	10,000	
Corals	4,000	20
Octocorals	1,000	3
Hydroids	2,000	23
Anemones	800–1,000	10
Nemerteans	900	14
Bryozoans	5,000	20
Ascidians	2,000	12
Molluscs	75,000	60
Echinoderms	6,000	
Ophiuroids	2,000	16
Echinoids and holothurians	1,400	11

vant to their own research. Most expressed an interest in selectively building, as well as maintaining, their institution's collection of the group or organisms they study.

Systematists who work at universities teach, of course, but public education is important to museum systematists as well. Most of them identify exhibits work, teaching, and answering questions from the public as part of their job. Under this obligation of service to the public comes the identification of specimens for other scientists. Almost all the systematists I talked with feel a certain amount of obligation to undertake this kind of work, but all of them consider it of secondary importance, agreeing that such work should not take up more than a small percentage of their time.

DIVERSITY OF RELEVANT GROUPS OF MARINE ORGANISMS

One reason the systematists surveyed feel an obligation to identify specimens for others is that they realize how few people can make accurate identifications of the organisms they study. This is partly due to the sheer diversity of the groups involved. In the first column of Table 1, I have listed conservative estimates of numbers of marine species for some groups of organisms in which interesting chemical structures or activities have been reported: algae, sponges, coelenterates (corals, octocorals, hydroids, and sea anemones), nemerteans, bryozoans, ascidians, molluscs, and echinoderms (ophiuroids, echinoids, and holothurians). In the second column I have listed a summary estimate, based on data provided by those interviewed, of the number of systematists actively working on those groups. This estimate is probably a liberal one. My definition of "active" does not include students working on Ph.D.s, or people who have done a dissertation on a group and have then gone into another area of research, but it does include people working part-time, and people who are retired, or amateurs, if they are regarded as by their colleagues to be consistently publishing or doing professional-level work.

There nearly 150,000 species in these groups alone, which include only a few of the plant and invertebrate phyla with marine representatives. Fewer than 200 people world-wide work on the taxonomy and systematics of these groups. That number includes people doing only occasional work, as well as people working on single families or genera. Moreover, some of these groups are considered by those who best know them to be greatly undersplit. For example, for bryozoans, the group I know best, the total number of species may be double that given here.

An idea of the significance of these numbers may be obtained by comparison with data for the sub-phylum Vertebrata, which has only 42,000 species, including some of the best-studied groups of organisms. For example, class Mammalia, with about 4,000 species, can boast about 50 specialists in the U.S. alone. The phylum Bryozoa, which on the basis of currently described species could be considered approximately equal in size, has only six U.S. specialists, and cannot claim 50 systematists studying Recent species in the world.

DIVERSITY RELATIVE TO SYSTEMATISTS

The taxonomic literature for almost all marine invertebrates, including those of interest here, is scattered and fragmentary. Taxonomic knowledge of any group in any geographic area is a function of whether in past or present any systematist has studied that group there. The areas of interest to natural products research are primarily tropical, the areas of the oceans that not only have the highest diversity of organisms, but, in most cases, have received the least attention from systematists. For example, for the Fifth International Coral Reef Congress in Tahiti, the organizers asked a number of specialists to evaluate the flora and fauna of French Polynesia (Richard 1985). Algae and molluscs from the region had been studied since the 18th century. Molluscs, with over 1,100 species listed, were considered well known (Richard *in* Richard 1985). The list for algae (Payri and Meinesz *in* Richard 1985) included 346 species, yet for some groups, particularly the Rhodophyta, it was considered to be far from complete. Corals appeared to be fairly well known, with 168 species listed (Pichon *in* Richard 1985). Sixty species of bryozoans were listed, but this included many unverifiable records, and the list was compiled from a few scattered collections considered by the author to be insufficient to show the richness of the fauna (d'Hondt *in* Richard 1985). For ascidians, data collection had just begun, and only a very incomplete list of 21 species was available (Monniot *in* Richard 1985). The list for echinoderms was also preliminary, with 30 species (Guille *in* Richard 1985). There were no reports for nemerteans, octocorals, hydroids, sea anemones, or sponges. It was after looking at a few reports like this that I decided to ask the taxonomists for their own estimates.

Table 2 gives estimates of the number of people working on these chemically important groups of organisms in eight geographic areas: East Coast (U.S.), West Coast, (U.S.), Caribbean, Indo-West-Pacific, Great Barrier Reef, East Pacific, Africa, and Antarctica. I chose the first two areas because I thought they would turn out to be relatively well known. The other areas were selected because they were tropical or otherwise environmentally stable. Much work has indicated that defensive toxicity is most prevalent in tropical regions (see review by Bakus et al. 1986), but it may also be common in other stable areas such as Antarctica (e.g., Winston and Bernheimer 1986).

The table refers to the number of specialists who study organisms from that geographic area (not to the area in which the specialists themselves live). As many people work on organisms from several geographic areas, the total number of systematists

TABLE 2. NUMBER OF SYSTEMATISTS STUDYING THE FAUNA OF SELECTED AREAS.

Group	U.S. east coast	U.S. west coast	Caribbean	Indo-Pacific	Great Barrier Reef	East Pacific	Africa	Antarctica
Algae	6	3	6	12	8	7	14	7
Sponges	3	3	5	5	4	0	3	2
Corals	3	3	6	7	—	3	1	1
Octocorals	1	1	1	3	—	1	1	2
Hydroids	1	4	3	6	1	0	1	1
Sea anemones	1	4	1	2	2	0	0	2
Nemerteans	2	3	2	1	2	0	0	1
Bryozoans	8	8	5	10	3	4	2	6
Ascidians	3	3	4	8	1	1	3	3
Opisthobranchs	3–4	7–8	5	5	2	7	4	4
Ophiuroids	2	2	3	5	1	2	0	1
Echinoids and holothurians	5	2	5	2	1	3	1	2

represented is much lower than would be concluded by totalling the numbers in the table. The main message of the table is that the number of systematists studying any of these groups in any of these areas is *very* low.

PERCENT OF FAUNA KNOWN

With the large numbers of organisms and the small numbers of systematists for each group it would not be surprising if a large number of species remained unknown. I asked respondents to estimate the percentage of the fauna known for their group in each area. Table 3 shows the results. All figures apply only to continental shelf or shallower depths. For sponges, figures are for 60 m or less and do not include the encrusting fauna, which is almost completely unknown. Estimates for corals are for reef depths only. Estimates for ophiuroids are for SCUBA depths. In deeper water, figures for all groups would decline drastically. For hydroids, the African estimate includes only South Africa, for other groups it is non-Mediterranean coasts. For some areas the people I interviewed either did not know or refused to guess. They are indicated with dashes. These estimates are, of course, just educated guesses, but they come from people who know each group well, and they are probably the best approximations possible at the present time.

It is clear that the U.S. coasts are fairly well known for most groups, with an overall average of about 80% of the fauna estimated to be known. Of course, some groups may only appear to be well known in an area. New techniques often lead to radical revisions. Where no one is actively working at present, and only older literature exists, the estimates may be misleading. For example, for nemerteans, the U.S. East Coast fauna includes many species described only on the basis of external morphology, which may not adequately represent the true diversity, based on modern methods of interpreting internal morphology.

The Caribbean appears better known than any of the following areas with about 70% of the fauna in the groups considered accounted for on average, and more than 75% thought to be known for six groups.

On the whole, Antarctica seems to be slightly better known than the non-Caribbean tropics with about 70% of the fauna in these groups accounted for. This is perhaps the result of two surges of collecting that took place there, the first between 1895 and 1925, and the second between 1958 and 1972. At both times taxonomists received support and encouragement in working up the collections that have seldom been available for work in other regions.

In the non-Caribbean tropics only 50–60% of the fauna is thought to be known. This represents an ocean area much larger than that surrounding Antarctica, which has still not received thorough study for most groups. I had included the Australian Great Barrier Reef as a separate area because I thought it might be better known than the Indo-Pacific region in general, but this does not seem to be true. The picture—from the point of view of the person seeking identification of a specimen from one of those areas—is bleak, for, even if a taxonomist can be found to look at it, it may well be undescribed.

TABLE 3. ESTIMATES OF % OF FAUNA KNOWN FOR SELECTED GEOGRAPHIC AREAS.

Group	U.S. east coast	U.S. west coast	Caribbean	Indo-Pacific	Great Barrier Reef	East Pacific	Africa	Antarctica
Algae	80	90	80	60	60	70	70	80
Sponges	75	75–80	60–65	60	60	50	40	50
Corals	95	80	95	70–80	70?	70–80	70	90
Octocorals	60–70	50	60–70	50	—	50	75	50
Hydroids	98	93	95	80–85	93	87	93	90
Sea anemones	—	90+	75	75	50	—	—	95
Nemerteans	70–80	50	20	20	20	20	20	20
Bryozoans	75	70	60	50	50	50	60	50
Ascidians	80	65–70	60	55	50	25–50	25–50	95
Opisthobranchs	80–90	80–90	60	20–30	40–50	40	70–80	40–50
Ophiuroids	85	90	80	60	—	—	—	70
Echinoids and holothurians	80	80	80	80	80	80	80	80

PROBLEMS OF IDENTIFICATION

Specimens of organisms in these groups may be difficult to identify even when they represent described species. Certain information about living specimens may be necessary for positive identification, specimens may be useless if improperly preserved or prepared, and identification techniques may be very time-consuming. The following section is included not as a guide to preparation, but to point out some of the problems for the groups considered.

Algae

Most algae can be identified from air-dried or pressed material, although a few genera must be preserved in liquid (5% buffered formalin). The systematist needs the entire plant, including holdfast. Reproductive structures are usually present and are visible with the naked eye (female structures) or a handlens (male structures), but unless they are included the alga may remain unidentifiable.

Sponges

For sponge identification a photograph of the living specimen is desirable; notes on color, form, and texture will also help. It is again important to collect the entire specimen. Otherwise, as one systematist put it, it is equivalent to identifying birds from feathers—in an area where the fauna is well known it can be done, but elsewhere it is impossible. Sponge specimens must also be fixed and preserved properly (see Ruetzler 1978). For species determination spicule mounts are most important and are relatively simple to prepare, but sometimes thick sections (to show the three-dimensional structure of the spongin fibers and relative position of spicules) or histological sections may be necessary. They may take days or weeks to prepare.

Scleractinians

Corals are one of the easier groups to prepare for identification. They may be preserved in alcohol or dried, as present taxonomy is based on skeletal characters. A good generic guide is available. However there are still many problems at the species level, some of which may be solved only by genetic work.

Octocorals

Octocorals may be dried or preserved in a non-acid preservative to avoid destruction of the spicules on which identifications are based. Spicule preparations are necessary and relatively easy, averaging less than half a day per specimen. These preparations are studied with the scanning electron microscope (SEM).

Hydroids

While most hydroids can be identified from preserved material, it is advantageous to have live material, and there are species that cannot be identified (sometimes even at the family level) without being cultured and followed through their entire life cycle.

Sea Anemones

Photographs of living animals are highly desirable. Specimens must be relaxed and dissected. Usually histological sections are necessary as well. Nematocyst smears are not diagnostic, but must be checked to make sure they are appropriate for the putative species. New techniques such as electrophoresis and life history work (especially the study of reproductive patterns) is increasing the number of species known.

Nemerteans

Nemerteans are probably the most time-consuming group considered here. To identify a species, a systematist must make serial histological sections. It may take up to two days to prepare one specimen (a fact that makes the low estimates of percent of fauna known in Table 2 much more understandable). For this group, also, it is important to know how the live animal looked either with a photograph or a drawing from life, with color notes.

Bryozoans

Bryozoans, like corals, are identified by the structure of zooid and colony skeletons. A dissecting microscope capable of magnifications up to 100 times is essential for preliminary work. A portion or all of the colony is bleached to remove tissue, stained with a dye to show pores and orifices, then examined. Difficult specimens are studied at higher magnifications (150–500×) in the SEM. Preparation time may take from 15 minutes to several hours per specimen. Studies of characters too small to be seen with dissecting microscopes, as well as genetic work, has convinced most specialists that the group has been greatly undersplit, and this means that the literature is misleadingly simple. It also necessitates museum study since old descriptions and illustrations cannot be relied on. In some cases even this is not enough to verify an identification, as most museum specimens cannot be examined by SEM.

Molluscs

Most molluscs are identified by their shells and opercula. However, radula preparations may be necessary for gastropods, and in some groups dissections of soft tissues may be important. For opisthobranchs, especially, photographs of the living animal are essential unless the collection is from a restricted geographic area in which the fauna is well known to the systematist.

Ascidians

Proper relaxation (using menthol) is important. Colonial forms with calcareous spicules must not be placed in an acid fixative or preservative that would destroy them. Many colonial forms have small zooids in which characters are difficult to determine without proper staining. Identification, even of larger forms, often requires dissection and examination of internal features under the microscope.

Ophiuroids

Ideally, ophiuroids for identification should be relaxed using $MgCl_2$ or $MgSO_4$ in seawater, spread in a tray of ethanol to hardern, and preserved in 70–80% ethanol. A specialist may need to dry specimens or to prepare material for SEM or light

microscope examination, but alcohol preserved specimens will usually suffice. Color notes or photographs of live specimens and samples of the substratum occupied by epizoic forms are very helpful. Juvenile specimens have not been well studied and are particularly difficult to identify. The problems with older literature—inadequate descriptions and illustrations—that plague all systematists apply to ophiuroid specialists as well.

Echinoids and Holothurians

Echinoids present few problems, as they can be dried or preserved in liquid. Holothurians must be narcotized and preserved in a non-acid preservative that will not erode the dermal ossicles used in identification. For holothurians the published literature is often poor and identification often requires extensive research.

TAMING SYSTEMATISTS

I hope that the preceding sections are convincing evidence of the diversity of the groups in question, the paucity of those qualified in their taxonomy, the gaps in knowledge of their distributions in regions where they might be of interest, and the difficulties involved in studying them. In spite of this, taxonomists/systematists feel some obligation to make identifications for others. I asked them what conditions they place on doing this kind of work, what makes them more or less likely to undertake identifications for others. Their answers were not unanimous, but there was some consensus.

Being able to keep type or representative specimens was important to most systematists interviewed. Of course such material must have been properly documented (Pomponi, this volume).

The second most important consideration seemed to be whether the material was directly related to the systematist's research—either to a group in which he or she was interested, or an area that was biogeographically important to his or her research. I did not ask respondents their ages, but I have a strong impression that willingness to look at material unrelated to one's own research area shows an inverse correlation with age. As one systematist put it, "When I first started it seemed as though much of the material sent to me led to interesting findings, a lot of papers. I don't know whether it's because I've seen more now . . . or whether it's truly the case that much of what's coming to me now is routine and less well-documented."

Several respondents said that they were interested in doing such work only in collaboration with others, for co-authorship. Others were willing to look at material as a courtesy, but stressed that they would look at it only if 1) they had been contacted beforehand and permission to send specimens had been granted, 2) the amount was not excessive, or 3) the material was easy (some said they would identify it if they found it easy, but send it back otherwise).

These considerations applied to work for colleagues as well as for companies. In doing identification work for commercial operations, financial reimbursement was also a factor, but not as strong a factor as might be expected. People who would not look at anything unrelated to their research did not change their minds if money was mentioned. In several cases, those who were willing to look at material for commercial organizations said that they were allowed to put any money they earned into a fund for their own research; others said they had requested and received various kinds of research support from such organizations. The chance to participate in cruises and collect in interesting areas was probably a stronger incentive than money to most systematists. If the people who need material identified could meet some of these criteria, it could enhance cooperation from systematists and result in increased satisfaction for both groups.

THE PERSISTENCE OF SYSTEMATICS

Returning to the quotation that begins this paper, it is clear that criticism of basic descriptive systematics has a long history. Yet systematics has persisted. I hope the tables presented here are convincing evidence that the need for basic description is still acute. Much more alpha taxonomy, collection, and description of organisms must be carried out before systematists can get on with the higher systematics of each group. This must be accomplished before the habitats are changed or destroyed. The situation in tropical reef environments, in particular, has been compared with that in tropical rain forests, where habitats and their inhabitants are disappearing much faster than we can catalog them. Ensuring the future of systematics is in the self-interest of those doing biomedical research on marine organisms. In order to have a significant underpinning for such research, a large amount of time must be devoted to developing the systematic framework for the groups of interest. It is not there at present, and it is probably not possible to utilize in biomedical research only those species that *are* well known.

Who will be doing this work? A number of reports have been devoted to plans for furthering systematics in this country (Steere et al. 1971; Irwin et al. 1973; Stuessy and Thompson 1981), but almost none of their proposals have been put into effect. In fact, for two reasons, the situation now may be worse than it was 25 years ago. One is that taxonomists as a group are aging. As in the academic profession in general, there are many people near retirement age. The most recent survey of the U.S. systematics community (Edwards et al. 1985) gave the modal age of these taxonomists as 41, and the mean age as 44, but this survey included undergraduate and graduate students, which skewed the age structure. I did not include students in my survey, and my impression is that the average age of practicing systematists is closer to 55.

The other problem is that there are few students going into the field. Some may be discouraged by the paucity of jobs. In invertebrate zoology there are few schools that encourage or even tolerate systematic work at the master's and doctoral level.

The future of systematic biology has received the attention of national committees and reached the editorial page of *Science* (Wilson 1985). It is obvious that the only long-term solution is strong support for systematics from the rest of the biological community. In the short run, for those trying to identify material, I have three suggestions. One is to take into account the considerations of taxonomists as given above and to try to work out arrangements that are mutually beneficial. Another is to utilize the skills of a second level of trained people—the biologists who worked on environmental surveys during the 1970s. Those who received good training on the systematics of one or more groups, at least for the geographic area in which they were working, may be available for taxonomic work and can often be contacted through local associations (e.g., the Southern Cal-

ifornia Association of Marine Invertebrate Taxonomists). The third suggestion is that more students be encouraged, perhaps by work/study programs in cooperation with large biomedical or natural products studies, to work with professional systematists. Some of the systematists I talked with are already involved in such programs. More might be interested in programs of that nature if they were available.

ACKNOWLEDGMENTS

This paper could not have been written without the assistance of those colleagues who courteously consented to answer my questions about their jobs and about the state of knowledge in the groups of organisms on which they work. My thanks go to all of the following: Dr. William K. Emerson, Mr. Walter E. Sage III, Dr. Ernst Kirsteuer, American Museum of Natural History; Dr. Daphne G. Fautin, Dr. Terry Gosliner, California Academy of Sciences; Dr. Francoise Monniot, Museum National d'Histoire Naturelle, Paris; Dr. F. M. Bayer, Dr. Stephen D. Cairns, Dr. Alan H. Cheetham, Dr. Linda Cole, Dr. Richard S. Houbrick, Dr. James N. Norris, Dr. David L. Pawson, Dr. Klaus Ruetzler, National Museum of Natural History, Smithsonian Institution; Dr. Gordon Hendler, Natural History Museum of Los Angeles County; Dr. Willard Hartman, Peabody Museum, Yale University; Dr. Patricia Mather, Queensland Museum; Dr. Dale R. Calder, Royal Ontario Museum; Dr. Ivan Goodbody, University of the West Indies.

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Maximizing the Potential of Marine Organism Collections for Both Pharmacological and Systematic Studies

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INTRODUCTION

The pioneering work of Bergmann (1949) on antibacterial agents from marine organisms has led to major research efforts over the past two decades to discover cures from the sea for human diseases. A more vigorous effort has been launched by several groups during the past five years, culminating in a recently expanded effort by the National Cancer Institute for *in vitro* screening of marine organisms against a large number of human cancers and viruses.

This emphasis on the collection and analysis of marine organisms offers both the chemist and the biologist a unique opportunity for research that can be maximized by care in planning, processing, and documenting the collection.

COLLECTION METHODS

A variety of collection methods are available, depending on the depth at which the organisms occur.

Marine natural products research began with organisms from intertidal and shallow subtidal environments where samples are easily accessible by wading and snorkelling. No special equipment or techniques are required. With minimal training, the collector can learn to recognize marine plants and animals that occur in different habitats, or certain groups of organisms that have been targeted for analysis. Samples can be collected manually by cutting, scraping, prying, or picking from the bottom. Sandy or muddy substrates can be sampled by coring, digging, and sieving.

The depth range of collections can be safely increased to approximately 37 m by using scuba. Of course, collectors must be trained to scuba dive, and additional safety precautions must be taken, particularly if deep dives are made routinely. There is the danger of diving accidents, such as air embolism and decompression sickness. As a precaution against such accidents, only experienced divers should be used for deep dives, and a recompression chamber should be located at or near the collection site so that immediate treatment is available if an accident occurs.

Most marine natural products research has been conducted on organisms collected from scuba depths. Advantages of this type of collection are the availability of abundant and diverse plants and animals in a variety of habitats, from coral reefs to kelp beds. Collectors can be selective, sampling targeted taxonomic groups and avoiding organisms that have been previously collected and are not needed for recollection. Manual collections enable one to sample only the amount needed for preliminary screens and bioassays, thus causing as little damage to the environment or organism as possible. Disadvantages include the possibility of diving accidents, and exposure of divers to hostile environments (e.g., low temperatures, poor visibility, strong currents) and dangerous marine organisms. At depths greater than 18 m, the amount of time a diver can spend on the bottom becomes a limiting factor, both physically and physiologically.

Within the last decade, emphasis has been placed on natural products chemistry of deep-water marine organisms, i.e., those occurring below maximum scuba depths. Two methods of sample collection are available, depending on the degree of reproducibility desired. Samples can be collected by dredging or trawling from a ship. The depth of collections is limited only by the size of the ship and the amount of cable on the dredge or trawl. Another advantage is the amount of material that can be collected from a productive site. It is not unusual for a dredge to be brought on deck with hundreds of kilograms of biological samples.

There are a number of disadvantages to dredging and trawling, however. It is often difficult to document accurately the depth or habitat from which the organism has been collected. Fragile samples can be damaged during collection, making taxonomic identification difficult or impossible, and often rendering the specimens unsuitable for bioassays. Samples are mixed together, making sorting difficult and contaminating individual specimens by the natural products of other samples, e.g., exuded pigments, mucus, and extruded viscera.

An alternative deep-water method is to use manned or unmanned submersibles. For the past four years, the manned Johnson-Sea-Link (J-S-L) submersibles from the Harbor Branch Oceanographic Institution (Fort Pierce, Florida) have been used for collection of specimens for pharmacological research (Fig. 1). The J-S-Ls consist of two chambers. A scientist and pilot sit in a clear, acrylic sphere that provides near-panoramic visibility, and a second scientist and submersible technician occupy the aft aluminum diver lock-out chamber with two observation portholes. The subs, having a certified depth rating to 915 m, are equipped with a multi-function manipulator arm for collection (Tietze and Clark 1986) (Fig. 2). Samples can be collected by a metal claw or a plastic Peterson-type grab. A suction device can be attached to the manipulator arm. Samples are deposited in 12 or 24 acrylic bins (Fig. 2), enabling the collector to separate organisms if necessary. An automatic data logger continuously records such variables as depth, temperature, and conductivity at programmed intervals. The submersibles are also equipped with 35-mm and video cameras for documentation of collections (discussed below).

There are some disadvantages with submersible collections. It often takes longer to collect samples with a manipulator arm than by hand, thus reducing the number or amount of samples collected per unit time. Organisms such as thin encrusting ones are difficult to collect in abundance with manipulator arms as presently configured.

Free-swimming submersibles are battery-powered, so bottom

* Current address.

FIGURE 1. The Johnson-Sea-Link II manned submersible (photo by T. Smoyer, HBOI).

time is limited to three to five hours. Tethered submersibles, or remotely-operated-vehicles (ROVs), that are powered by shipboard generators can theoretically collect until all bins and baskets are filled with samples.

Degree of visibility can be limiting, ranging from 360° from the front sphere of the J-S-Ls, to smaller viewing angles in other manned submersibles. With an ROV, all observations are made through video cameras, so the degree of visibility is dependent on the number and position of cameras on the sub.

Depth is limited to the certified rating of the manned submersible and the depth rating and amount of tether on an ROV. Some manned submersibles are rated to greater depths than the J-S-Ls, and their work packages can be configured for biological collections. Collection capabilities of these submersibles vary, however, with the type of manipulator arm and sample storage capabilities.

Regardless of collection method, accurate data must be kept on site location to enable the researcher to return to the same locality for recollections, if necessary. At the very least, latitude and longitude should be recorded, along with names and descriptions of near-by land masses, if any. Most oceanographic research vessels rigged for trawling, dredging, or submersible operations are equipped with sophisticated navigational equipment. Collection site data are normally recorded by the vessel operator, and should be transcribed from the ship's log into field notes.

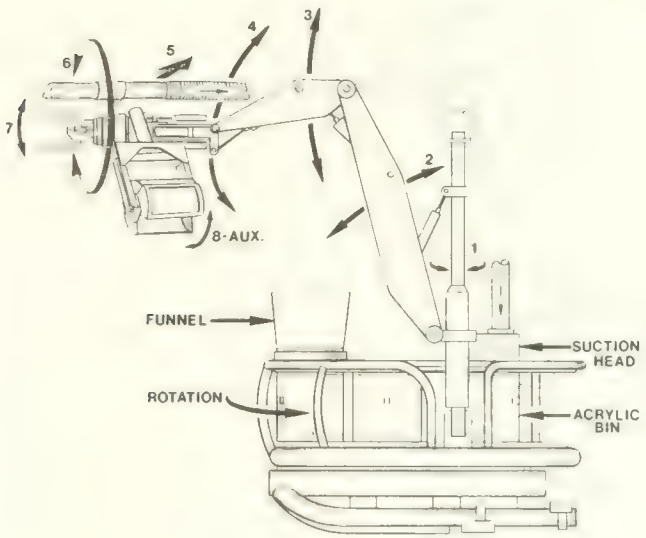

FIGURE 2. Detail of multi-function manipulator arm, suction device, and work platform with acrylic storage bins on the J-S-L submersibles (courtesy R. C. Tietze, HBOI).

SAMPLE PROCESSING

Sample processing refers to treatment of samples between the time they reach the field laboratory and the time they are preserved. If there will be a delay of more than a few minutes between the time samples are collected and the time they are processed, they should be separated into individual plastic bags or buckets and bathed in seawater, preferably at the same temperature at which they were collected. If this is not feasible, they should be stored on ice. If there will be a delay of several hours, samples should be frozen with dry ice to prevent deterioration.

Samples must first be sorted, matching specimens with any field notes that were taken at the time of collection. If taxonomists are present, some samples can be identified; others may be tentatively identified and grouped. Samples not obviously the same species should be kept separate. Collectors should familiarize themselves with characters used by taxonomists for identification of species. These characters vary with the taxonomic group. Some characters, such as color, are lost or changed after the organism is preserved. It is particularly important for subsequent taxonomic identification that collectors recognize and record these characters. If arrangements have been made for identification of samples, consult the systematists *before* collecting the samples and request a list of key characters, proper relaxation, fixation, and preservation techniques, and references to pertinent taxonomic literature.

All samples should be labelled in the field. Numbering systems are as varied as the number of collectors, and can incorporate date, location, or some other parameter. For example, the number 30-IV-87-01-001 refers to 30 April 1987, collection #01, sample #001. Numbers can be partitioned into phyla or kingdom, e.g., animals from 001 to 099, and plants from 101 to 199, to facilitate database searches. Waterproof paper and stick-on labels can be numbered manually or by computer.

If a large number of samples will be collected, extracted, and screened, use of a bar-code labelling system could facilitate accurate, rapid logging and inventory of the samples. A bar-code reader, interfaced with a computerized data base, can be used to maintain accurate records on each sample after processing.

Other optional steps in sample processing include preparing an extract of the sample, weighing the sample, and cutting it into smaller pieces for more effective preservation or storage.

VOUCHERS

An important part of sample processing is the preparation of a reference sample, or voucher. A voucher enables a specialist to identify the sample and to provide a reference for recollection of the same species, if necessary.

For identification of a sample to the species level, the voucher must retain all attributes that make it unique. For some taxa, an entire organism must be preserved. For others, such as

sponges, a representative cross-section is often adequate for identification, particularly if good field notes and photographs of the intact organism are available. It is advisable to include a taxonomist in the field collection team to supervise the preparation of vouchers. Those processing samples should be trained in the preparation of proper vouchers for each taxon. If neither of these options is feasible, a complete specimen should be preserved as a voucher. If a sample consists of several individuals with morphological variations among them, the voucher should contain representative samples of each morphological type.

The value of a voucher collection cannot be over-emphasized. There are many examples in the natural products literature where samples have been improperly identified. If there are no reference samples with which an accurate identification can be made, the research results are of little value to either the chemist or the systematist. At the time a paper is submitted for publication, or a patent application or record of invention is filed, the sample number and location of the voucher specimen should be indicated. Ideally, the voucher sample should be deposited in a museum or archival institution. In practice, this is rarely done for fear of disclosing proprietary information to competitors. Once the patent/paper describing the activity and the species has been granted/accepted, however, the voucher should be made available to the scientific community. Vouchers of samples with no bioactivity, that are of no further interest to the chemist, should be routinely deposited in a museum.

Before any paper is submitted for publication, or patent application or record of invention is filed, identifications should be verified. Taxonomic revisions could have occurred since the time the sample was originally identified, or the systematist might have reason to change the original identification. At the very least, correct spelling of the scientific name must be verified by the systematist. Many scientific names are similar, with related taxa often having variations of a parent taxon name. Names can be easily confused or misspelled during any stage of manuscript preparation or publication. Galley proofs should be carefully checked for misspellings.

Sample Preservation

Once an adequate voucher has been taken from each sample, it must be transferred to an appropriate fixative and preservative. Many organisms must be anaesthetized so that structures necessary for identification do not contract during preservation. There are a number of manuals (e.g., Lincoln and Sheals 1979) that describe relaxation, fixation, and preservation methods for the various groups of organisms. Sturdy plastic bags, glass jars, or clear plastic jars are suitable containers for vouchers. Selection of a container will depend on the amount of space available for storage. Plastic bags should be heat-sealed, and jars should have lids that prevent leakage during transport. Voucher samples can be stored at room temperature.

A label, on waterproof paper that has a plastic-coating or high fiber content, should be placed *inside* the voucher sample container. Pencil or waterproof India ink will not wash off the label in preservative, but ball point pens or indelible markers should not be used. Labels can also be typewritten, using a nylon film or Mylar ribbon. A cloth ribbon should not be used, because the ink is not water- and solvent-resistant.

For samples to be assayed in the field, a small subsample should be extracted. The remainder of the sample should be weighed, if possible, and preserved for subsequent chemical and biological analysis. Field preservation techniques vary, depending on the class of chemical compounds being studied. One common field preservation method is freezing at −20°C. Once the samples have been returned to the laboratory, they can be lyophilized or preserved in some other manner.

When frozen samples are no longer of interest, one should consult a taxonomist or museum curator before discarding the specimens. Some thawed specimens can be preserved or dried, and may become valuable as reference material.

DOCUMENTATION

Adequate documentation of the collection is essential to both chemist and systematist. The chemist can use such data to predict trends in bioactivity, for example, in relation to biogeography, depth, taxon, or morphology. Notes in the field log, such as presence of eggs or the observation of a particular feeding behavior, can provide the biologist with useful reproductive and behavioral data.

Data manipulation can be facilitated by using a computerized data base. For example, the National Cancer Institute (NCI) provides its collection contractors with a program in *dBase III Plus* (Ashton-Tate, Torrance, California) for the input and formatting of data for later transfer to the NCI's DEC 10 mainframe computer Drug Information System. Fields for collection and site data forms for recording information (Fig. 3, 4) were defined by Janice E. Thompson (NCI, Natural Products Branch) and myself. To facilitate data entry, codes were defined for collection and site variables (e.g., habitat, substrate, toxicity, abundance, color, morphology, phylum). These codes are available to users of the NCI's Drug Information System.

The sample data form (Fig. 3) provides a guide for logging field notes during collection and sorting. Most morphological data can be entered in the field. Some taxonomy data (e.g., phylum) can be entered in the field; the remainder are usually entered at the time of sample identification. Relaxation, fixation, and preservation data are entered at the time the voucher specimen is prepared. A field is provided for general notes not appropriate to any other field, or to expand on coded entries.

A field notebook with waterproof paper can be prepared with column headings corresponding to some of the data fields appropriate for field entry. These notes are then transcribed into the *dBase III Plus* program after samples have been processed. A portable computer with a 20-megabyte hard disk is adequate for data entry in the field.

Using the program, data can be indexed, listed, or sorted on criteria such as taxon, weight, bioactivity, or depth. This is very useful if one wants to know, for example, which sponges of a certain species have antiviral activity, or if that activity varies with depth or location. Using this program, one can also generate labels and print records of all data entered.

Photography

Photographs of the samples, particularly in situ photographs, are invaluable both to the taxonomist and to the collector if the samples must be recollected. As discussed above, this is not

COLLECTION # _____ SPI SITE # _____ SPI SAMPLE_____

DATE __/__/__ DEPTH(m) ___.__

HABITAT _____ SUBSTRATE _____

HAZARD CODE _____ ORGANISM PARTS _____

ABUNDANCE ___ EXTERNAL COLOR _____ INTERNAL COLOR _____

MUCUS ___ ODOR _____ MORPHOLOGY_____

EPIBIONTS _____

EPIBIONT COVER _____ SYMBIONTS_____

CYANOBACTERIA _____ ZOOXANTHELLAE _____ ZOOANTHIDEA _____

ASSOCIATIONS/INTERATIONS_____

PHYLUM _____ CLASS _____

ORDER _____ FAMILY _____

GENUS _____ SPECIES_____

AUTHORITY_____

LITERATURE_____ IDENTIFIED BY _____

LOCAL NAMES_____

BIOACTIVITY_____

RELAXATION _____ FIXATION _____ PRESERVATION ___

OTHER R/F/P _____ WET WT(g)_____

GENERAL NOTES_____

FIGURE 3. Data input form for sample data (courtesy National Cancer Institute, Natural Products Branch).

SPI SITE # _____

LATIDUDE _____ LONGITUDE _____ GEOGRAPHIC REGION ____

LOCALITY _____

DISTANCE FROM SHORE (m) _____

MARINE ENVIRONMENT _____

SHORE ENVIRONMENT _____

TEMPERATURE RANGE C _____ VISIBILITY RANGE (m) _____

SALINITY (PPT) _____ CURRENT (kts) _____ AVG WAVE ACTION ___

GENERAL NOTES _____

FIGURE 4. Data input form for site data (courtesy National Cancer Institute, Natural Products Branch).

routine during dredging and trawling operations, although sleds with 35-mm and video cameras can be attached to dredges and trawls. Submersibles are normally equipped with both 35-mm and video cameras. Videotapes can often provide more information to the taxonomist than a still photograph, and are helpful for recollections.

If *in situ* photographs are not feasible, surface photographs can suffice, particularly if care is taken to include taxonomic details. Samples should be photographed against a neutral background, and care should be taken to eliminate shadows. If the sample is fragile or collapses when removed from water, it should be photographed in a tank of clean seawater. Surface photographs may not be feasible in the field. As an alternative, voucher samples can be photographed after the specimens are transported back to the laboratory.

Photographs and collection data should accompany samples sent to taxonomic specialists.

OTHER CONSIDERATIONS

Site selection is a very important consideration and could have a major impact on the success of a collection expedition. It is advisable to consult with marine scientists who have worked in a particular area before planning an expedition. It is also often worthwhile to send an advance field team to the proposed collection site to meet with local officials and those who make their living from the sea (commercial fishermen, dive charter operators). They can often provide information that is not available in a book or on a nautical chart.

Many governments require a collecting permit before any samples can be removed. Be sure to inquire from appropriate officials well in advance of any planned expedition. Processing collection requests can take as long as one year for some countries. Consult with the United States Department of State, Bureau of Oceans and International, Environmental, and Scientific Affairs for advance notice requirements for foreign research permits.

Collection of samples for pharmacological research requires planning and care during each phase of the collection. The purpose of this paper is to provide guidelines for a successful collection, resulting in samples useful to both the chemist and biologist.

ACKNOWLEDGMENTS

Much of the practical information on collections resulted from collaboration during field collections with Dr. J. E. Armstrong, SeaPharm/Harbor Branch Oceanographic Institution (HBOI), and ship and submersible crews of the R/Vs *Sea Diver, Seward Johnson, Edwin Link,* and Johnson-Sea-Links. Partial support has been provided by the National Cancer Institute (Contracts NO1-CM-67919 and NO1-CM-67967).

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Screening to Detect Biological Activity

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INTRODUCTION

In this Symposium on the biomedical importance of marine organisms, the subject assigned to me is especially pertinent, since it is screening that must initially establish biomedical activity. While screening has also been employed to identify other activities—antifouling (Rittschof et al. 1986), ichthyotoxicity (Bakus et al. 1986), shark repellency (Zahuranec 1983), and pesticide activity (Crawley 1988), to name a few—I shall, in keeping with the Symposium's theme, limit this discussion to activities of use in human medicine.

The use, or more accurately, the potential use, of marine natural products in human medicine has been reviewed extensively elsewhere (i.e., Krebs 1986; Rinehart 1988), often under the catch phrase "Drugs from the Sea" (Freudenthal 1968; Youngken 1970; Worthen 1973; Webber and Ruggieri 1976; Kaul and Sindermann 1978). In spite of this literature, the history of systematic screening in the marine area is relatively short. Serious efforts to locate antimicrobial activity were initiated by Burkholder (Burkholder and Burkholder 1958) and by Nigrelli (Nigrelli 1952; Nigrelli et al. 1959) in the 1950s. The National Cancer Institute (NCI) maintained an extensive screen for antitumor activity from the mid-1960s to 1985 (Hartwell 1971, 1982; Boyd et al. 1988). Systematic antiviral screening began with our 1978 *Alpha Helix* Caribbean Expedition (AHCE 1978) (Rinehart et al. 1981*b*). During the late 1970s, the Roche Institute of Marine Pharmacology (RIMP) screened for a variety of biomedical activities (Baker 1976*a, b*; von Berlepsch 1980). The 1980s have seen a rekindling of interest in screening for marine-derived pharmaceuticals, both in industry, where at least one company—SeaPharm—is devoted wholly to marine-derived drugs, and in academia (Jefford 1988). This renewed interest is, of course, the rationale for this Symposium.

TYPES OF SCREENS

General

I shall deal shortly with some specific screens, but it should be noted at the outset that screens generally fall into two categories. The first type can be characterized as directed, in that a specific bioactivity is sought and a large number of extracts of marine species are tested for that specific activity. When the desired activity is found, attempts are made to isolate and characterize the responsible compound or compounds. This type of screen, directed toward a specific activity, is, of necessity, biology-driven. The NCI, Alpha Helix antiviral, and SeaPharm screens are examples.

The second type of screen is initially not directed toward a specific bioactivity. Rather, marine species are extracted and the compounds obtained are separated, isolated, and characterized. Then, the isolated compounds are screened for a variety of bioactivities. This non-directed screening, which is thus chemistry-driven, can also be described as serendipitous or *ex post facto* screening. The RIMP screening was of this nature, as is the Santa Barbara screening (Jacobs et al. 1985). Obviously, combinations of the two screening types are also possible.

Primary Screens

The screens described in the previous section are primary screens, designed to provide an initial identification of bioactivity. Both directed and non-directed primary screens can be carried out on samples after their return to the home laboratories of the screener. Directed screening, however, often has the advantage of being amenable to on-site screening at remote field locations. Chemistry-driven screening requires extensive chemical separations as the first step and is not amenable to on-site primary screening.

Extensive on-site screening was first introduced during our 1974 *Alpha Helix* Baja Expedition (AHBE 1974) (Hager et al. 1976; Rinehart et al. 1976; Shaw et al. 1976) and expanded during AHCE 1978 (Rinehart et al. 1981*b*). It provides a number of significant advantages. First, screening is carried out on organisms immediately after collection, offering the greatest chance for a positive result. Examples abound of the loss of bioactivity observed in the field, either by chemical or biological decomposition during the return to the home laboratory. A case in point is that of our eudistomin research (Rinehart et al. 1987*b*). The initial extract of *Eudistoma olivaceum* was highly antiviral when tested on-site during AHCE 1978, but was essentially inactive when tested in Michigan following our return. Had the initial screening been performed there, the activity would never have been discovered. A second advantage of on-site (field) screening is that the species identified as active can be targeted immediately for re-collection at the original site or nearby locations.

It may be well at this juncture to record some of the characteristics sought in a primary screen. Ideally the screen should be sensitive, since bioactive components are often present in trace amounts, and not overly selective, since it is easier to narrow a list of candidates than it is to generate new entries. It should be quantitative, or at least semi-quantitative, in order to compare candidates. Ideally, too, the screen should be fast, both in requiring little actual time and in providing a short turnaround time. It should be reliable, and for field use it should be simple and rugged.

Secondary Screens

Once a candidate drug has been identified by a primary screen, secondary screens are employed to define the drug's value. Secondary screens are usually in the same therapeutic area and serve either to expand the spectrum of activity or to evaluate the primary activity. For example, if anti-*Herpes* activity has

TABLE 1. BIOACTIVITIES OF TUNICATE (*TRIDIDEMNUM* SPECIES) SAMPLES.

	AHCE sample#								
	55	241	580	614	634	676	738	484	755
Antiviral assays									
Shipboard HSV-1[a]	+3	±2	NT	NT	NT	NT	NT	±	+
Secondary testing[b]									
DNA viruses									
HSV-1	2/4	1/2	2/4	0/4	4/4	0/4	4/4	4/4	0/4
HSV-2	2/4	1/3	2/4	0/4	4/4	0/4	4/4	4/4	0/4
Vacc	1/4	0/3	2/4	0/4	4/4	0/4	4/4	4/4	0/4
RNA viruses									
PR8	2/0	2/3		4/0	4/4	4/0	4/4	4/4	4/4
HA-1	1/3	3/4	2/4	2/4	4/4	2/4	4/4	4/4	0/4
COE	2/3	3/4	2/4	2/4		2/4			0/4
E.R.	2/4	2/4	2/4	2/4	4/4	2/4	4/4	4/4	0/4
Cytotoxicity									
Shipboard CV-1[c]	35	49	NT	NT	NT	NT	NT	0	70
Secondary testing[d]									
L1210, ID_{50} (μg/ml)	0.015	0.16	0.052	0.26	NT	0.030	NT	0.20	0.90

[a] Number of assays showing strong (+) or weak (±) inhibition of *Herpes simplex* virus, type 1 (HSV-1) during the *Alpha Helix* Caribbean Expedition (AHCE) 1978. Sample was 100 μl of a 20-ml methanol-toluene (3:1) extract of 2 g of sample. NT = not tested.

[b] Activities expressed as the relation of zones of cytotoxicity to zones of virus inhibition (zones of inhibition: 1 = 1 to 10 mm, 2 = 10 to 20 mm, 3 = 20 to 30 mm, and 4 = 30 to 40 mm) for 20 μl of solutions containing 1 mg/ml of sample. HSV-1, HSV-2 (*Herpes simplex* virus, types 1 and 2) and Vacc (vaccinia virus) all grown in primary rabbit kidney cells; PR8 (influenza virus) grown in embryonic chick kidney cells; HA-1 (parainfluenza-3 virus) grown in Hep-2 (human epidermal carcinoma) cells; COE (Coxsackie A-21 virus) and E.R. (equine rhinovirus) grown in ML (a variant of HeLa cervical carcinoma) cells.

[c] Zone of inhibition of CV-1 cells, extrapolated to 100 μl of a 20-ml methanol-toluene (3:1) extract of 2 g of sample.

[d] Inhibition of L1210 cell growth in culture. Cells (5×10^5 cells/ml) were incubated continuously for 3 days at 37°C with the sample at various concentrations. Cell numbers were then determined with a Coulter Counter (Coulter Electronics, Hialeah, Florida). Sample concentrations required for 50% inhibition (ID_{50}) were obtained by plotting the logarithm of sample concentration against percent inhibition of cell growth.

From Rinehart et al. 1981*a*, *b*, and 1983.

been identified by an *in vitro* primary assay (see below), secondary screens might consist of *in vitro* assays against other DNA viruses or a number of RNA viruses (Table 1). Alternatively, the *in vivo* efficacy of the candidate drug against *Herpes* infections might be examined. Such secondary screening usually includes repetition of the primary assay, if that was carried out in the field, as well as repetition of the primary screen at different concentrations. Secondary screening can be carried out either at the home laboratory or at a commercial testing laboratory.

EXAMPLES OF CURRENT SCREENS

ANTIMICROBIAL SCREENING

The first widespread screening carried out on marine organisms, as noted above, involved antimicrobial assays, which are, not coincidentally, probably the simplest. Disk assays in Petri dishes are generally rugged and reliable. Ideally a spectrum of test microorganisms is employed, even in the field, including a Gram-positive bacterium (usually *Bacillus subtilis* or *Staphylococcus aureus*), a Gram-negative bacterium (usually *Escherichia coli*), and one or more fungi (a *Penicillium* or an *Aspergillus* strain, *Saccharomyces cerevisiae*, or *Candida albicans*). An aliquot of the test extract is added to a filter paper disk on a lawn of the growing microbe and a positive assay is measured in terms of the diameter of a clear zone of inhibition of microbial growth (Fig. 1). Secondary screens include a much broader spectrum of bacteria and fungi, including resistant strains, as well as representatives of anaerobic microorganisms (*Clostridium* or *Bacteroides* strains). In addition, the activities are more accurately measured, in solution, as minimal inhibitory concentrations (MICs). *In vivo* studies of bacterial or fungal infections in mice would normally follow. However, antimicrobial compounds from marine sources have never been active enough to compete with classical antibiotics from microorganisms.

ANTIVIRAL SCREENING

Standard antiviral screening usually involves growing a mammalian cell line (such as CV-1 monkey kidney cells) in wells and infecting the cells with the test virus (for example, *Herpes simplex* virus, type 1) (Schroeder et al. 1981). A small filter paper disk is then impregnated with a measured solution of the extract or compound to be tested. After incubation, a dye is added to stain the cells and the area around the disk is inspected for two types of activity. Cytotoxicity is judged by lack of staining due to cell death. Where cells survive, antiviral activity is judged by absence of the small white viral plaques due to viral replication (Fig. 2). Since *Herpes* is a DNA virus, an RNA virus is frequently tested as well. In the past, vesicular stomatitis virus (a sheep virus) has been employed, both at the University of Illinois and at SeaPharm. More recently, an A59 Corona virus (a mouse hepatitis virus) has been added at SeaPharm as a second representative RNA virus.

Secondary antiviral screening normally includes testing the extract or compound against a battery of DNA and RNA viruses (Table 1) followed by quantitative determinations of activity (Fig. 3). *In vivo* tests in mice then follow, employing the viruses used for *in vitro* screening. For activity against DNA viruses, tests against vaginal *Herpes* (Table 2), skin *Herpes*, *Herpes encephalitis*, or *Herpes keratitis* are used. Activity against Rift Valley fever is illustrative of an RNA virus.

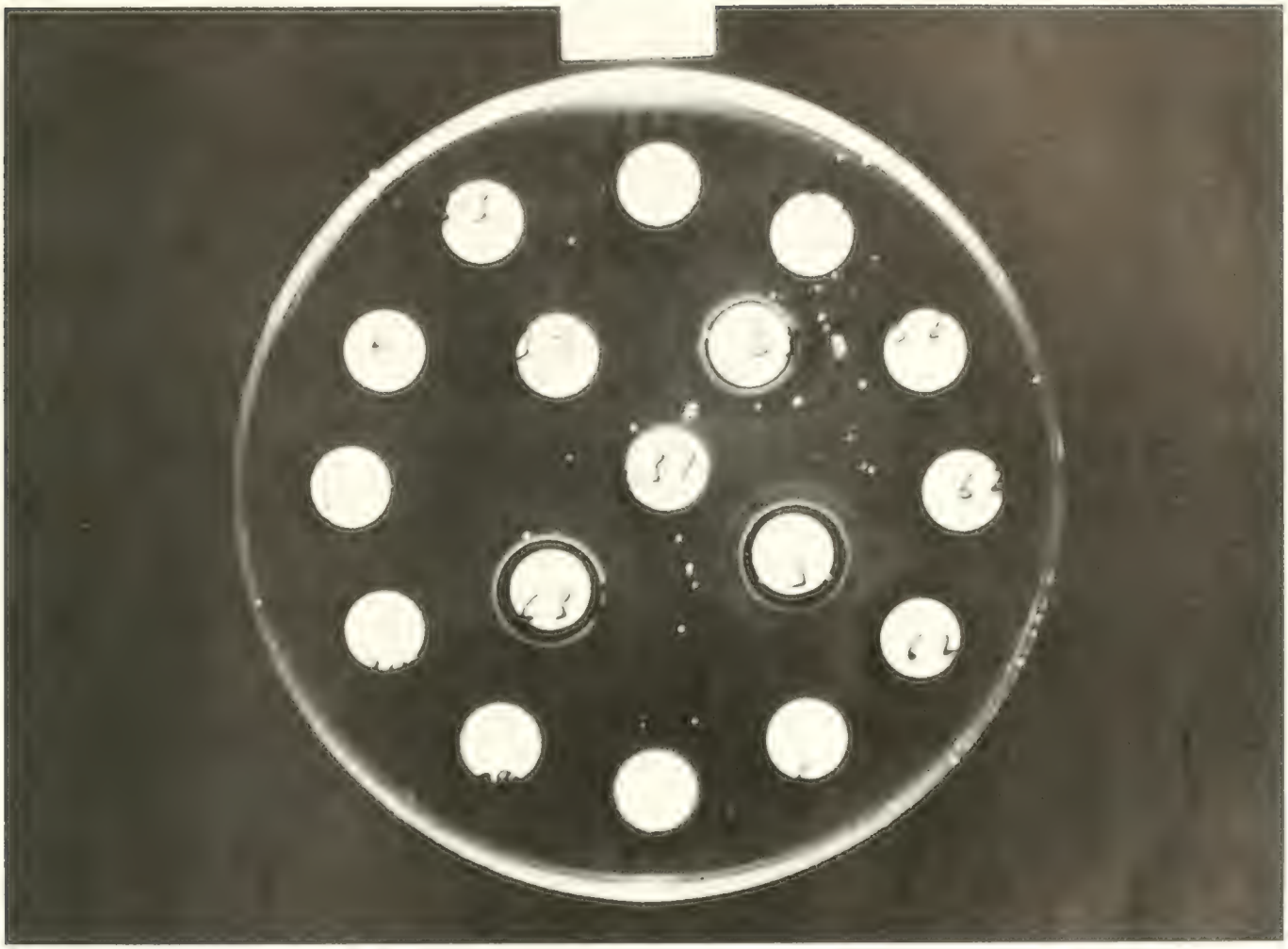

FIGURE 1. Antibacterial screen versus *Bacillus subtilis*. Active extracts are identified by clear zones surrounding the extract-impregnated disks. S = tetracycline standard.

ANTITUMOR SCREENING

A variety of primary screens have been employed as predictors of antitumor activity. In extensive screening of marine organisms from the 1960s to 1980, the National Cancer Institute initially used an *in vivo* mouse assay as the primary screen. This involved injections with L1210 or P388 leukemia cells, with success measured in percent life extension versus control mice, a so-called T/C rating (Hartwell 1971, 1982; Boyd et al. 1988). While the information derived was valuable, the assay was slow for a primary one, and it was especially cumbersome for following activity during isolations.

In 1975 the primary assay was shifted to an *in vitro* cytotoxicity assay employing P388 leukemia cells (Suffness and Douros 1982; Boyd et al. 1988). In our own testing we have used *in vitro* cytotoxicity assays employing either P388 or L1210 leukemia cells. Both assays have been carried out successfully on shipboard by SeaPharm.

A cytotoxicity assay employing non-malignant mammalian cells has also been used as a rough primary screen for antitumor activity in field testing on both University of Illinois and SeaPharm expeditions (Rinehart et al. 1981*b*). Because the antiviral assays are carried out in mammalian cells, cytotoxicity toward these cells (usually CV-1 cells) is detected simultaneously with antiviral activity. While cytotoxicity to normal cells is ultimately undesirable, it can provide leads to antitumor compounds (cf. didemnin B below).

TABLE 2. PROTECTION OF FEMALE MICE FROM GENITAL HSV-2 INFECTION BY DIDEMNINS A AND B.[a]

Treatment	Drug concentration (mg/ml)	Death/total	Survival (%)	Mean death (day)
Saline	0	13/14	7.1	6.5
DMSO (10%)	0	14/14	0	6.7
Didemnin A	1.0	6/14	57.1[b]	9.3[b]
	0.1	14/14	0	6.9
Didemnin B	0.23	4/14	71.4[b]	8.0[b]

[a] Mice infected with intravaginal inoculation of HSV-2 were treated intravaginally 3 times per day for 3 days with 0.1 ml of drug, beginning 1 hr after inoculation. Illnesses and deaths were recorded daily for 21 days. HSV-2 (strain 35D 9.0×10^4 PFU/0.1 ml) was inoculated at T

[b] Probability: $P < 0.01$

From Rinehart et al. 1982 and 1983

FIGURE 2. Anti-viral screen versus *Herpes simplex* virus, type 1. Absence of white viral plaques indicates an anti-viral extract. Absence of staining of the cells by the dye indicates a cytotoxic extract.

Two other assays have been employed in screening for specific types of antitumor candidates. One of these is the biochemical prophage induction assay (BIA) (Elespuru and White 1983). This assay employs a genetically deficient *E. coli* strain in which the β-galactosidase gene is repressed. Active extracts interact with the strain's DNA, allowing expression of the gene by hydrolysis of a β-galactoside, which can be measured colorimetrically. While it is sensitive, simple, and fast, the BIA screen identifies only DNA-interactive antitumor agents.

The second limited screen involves inhibition of cell division of sea urchin eggs (Jacobs et al. 1981). This assay has the distinct advantage in the field that sea urchins are nearly always available. It gives positive results, however, only for the limited class of antitumor agents that inhibit microtubule assembly. Thus, it fails to detect most antitumor compounds in clinical use.

For the future, the National Cancer Institute plans to screen potential anticancer candidates *in vitro* against a battery of human tumor cell lines including lung, colon, breast, renal, prostate, melanoma, ovarian, and CNS tumors (*The New York Times* 1986; Boyd et al. 1988; Suffness and Thompson, this volume). The hope is that agents will be identified that show selective activity against these cancers, which are the most common human tumors.

For secondary antitumor screens, *in vivo* testing is employed. This has traditionally used the P388 leukemia assay, illustrated by results with didemnin B (Table 3), followed by tests with other tumors (for example, B16 melanoma).

Immunoregulatory Screening

The observation of *in vitro* immunosuppression by didemnin B at a concentration considerably lower than that of cyclosporin A (Montgomery and Zukoski 1985) stimulated a search for additional immunomodulators in the marine environment using two types of primary screens. The first of these involves a comparison of T-cell and B-cell mitogenesis in the presence and absence of test extracts, with different stimulants being employed for the two types of cells—concanavalin A for T-cell mitogenesis and lipopolysaccharide for B-cells (Montgomery and Zukoski 1985). The second type of immunoregulatory test employs a mixed lymphocyte reaction (MLR) (DeWolf et al. 1980) in which lymphocytes from two genetically different mice are mixed, thus stimulating an immune response that can be enhanced or suppressed by the test extract. Both assays are measured by radioactive thymidine incorporation and the MLR can be automated by colorimetric measurement of a dye.

In secondary testing for immunoregulation *in vivo*, one test involves the graft-versus-host reaction in which spleen cells from

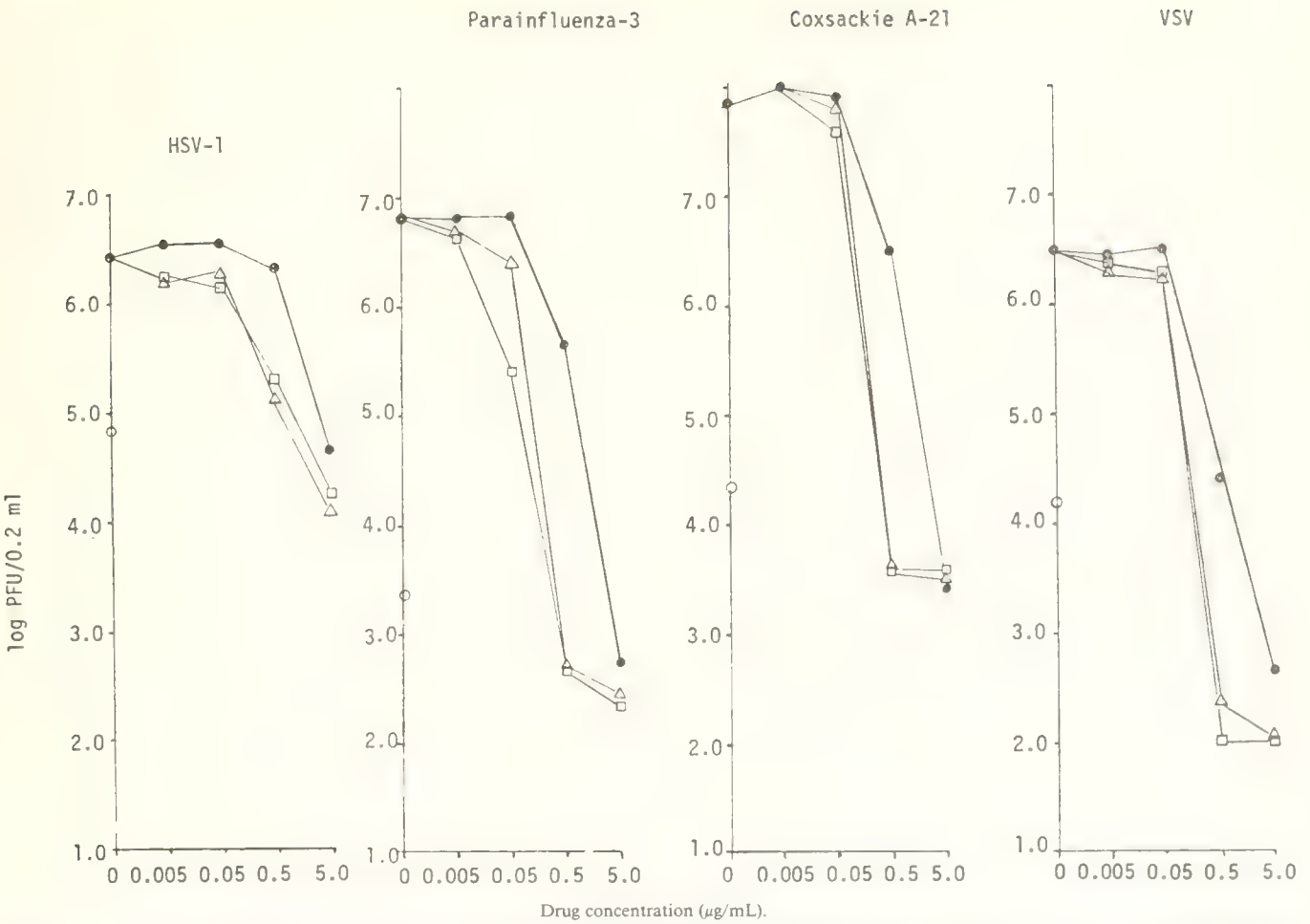

FIGURE 3. Virus yields from cell cultures infected with *Herpes simplex* virus, type 1 (HSV-1), vesicular stomatitis virus (VSV), Coxsackie A-21 virus, or parainfluenza-3 virus when treated with different concentrations of didemnins A (●), B (□), C (△). From Renis et al. 1981, and Rinehart et al. 1982.

one strain of mouse are introduced into a second strain, and the increase in spleen size in the second strain is measured gravimetrically (Montgomery and Zukoski 1985).

CARDIOREGULATORY SCREENING

Observations of cardiostimulation by marine organisms (Norton 1981; Alsen 1983) have prompted some systematic screening in our laboratory of marine extracts with the hope of finding a safe heart stimulant (Traeger 1985; Catlow 1986). A useful primary screen in a stable laboratory involves the excision and grinding of an infant mouse heart; the heart cells continue to beat in synchrony for some time, and the frequency and amplitude of the beating can be recorded (Harary and Farley 1960). An alternative screening assay, which we have employed in field testing as well as in our home laboratory, involves attaching a whole excised frog heart to a pair of electrodes and a recorder from which both amplitude and frequency of beating can be measured. While results of the frog heart assay are obtained rapidly, a relatively small number of samples can be processed each day, the total number being limited by the number of frogs available.

TABLE 3. ANTITUMOR ACTIVITY OF DIDEMNINS AGAINST P388 LEUKEMIA.[a]

Didemnin	Drug: Route	Schedule	Dose (mg/kg/day)	T/C[b] (%)	Body weight change (g)
A	i.v.	day 1	64	118	−1.3
			32	107	−1.3
	i.p.	day 1	64	91	−2.2
			32	103	+0.1
		days 1, 5, 9	32	107	−0.8
			16	102	+1.0
B	i.v.	day 1	4	T[c]	T
			2	109	−2.8
			1	104	−1.7
	i.p.	day 1	2	T	−2.5
			1	104	−1.4
		days 1, 5, 9	1	94	−1.9
			0.5	106	−0.5

[a] Tumor was inoculated (i.v.) at 10^6 cells/mouse.
[b] Median death of untreated P388 leukemia-bearing animals (control) = 8.5 days.
[c] Toxic.
From Li et al. 1981.

TABLE 4. ANTIMICROBIAL AND ANTIVIRAL ACTIVITIES AND CYTOTOXICITY IN PHYLA ASSAYED DURING THE *ALPHA HELIX* CARIBBEAN EXPEDITION 1978.

Phylum	% species active[a] (number of species examined)[b]						
	Overall antimicrobial	*E.c.*	*B.s.*	*S.c.*	*P.a.*	HSV-1[c]	CV-1[d]
Porifera	(187)	14	41	19	11 (138)	14 (180)	62 (186)
Cnidaria	(70)	4	26	7	2 (66)	17 (69)	56 (70)
Ectoprocta	(1)	100	100	0	0	0 (1)	0 (1)
Mollusca	(20)	5	15	0	0 (17)	0 (21)	33 (21)
Annelida	(3)	33	0	0	0	0 (3)	0 (3)
Arthropoda	(6)	0	0	0	0	0 (6)	0 (6)
Echinodermata	(36)	0	3	50	26 (27)	16 (36)	72 (36)
Chordata	(27)	15	37	15	14 (22)	23 (26)	70 (27)
Cyanophyta	(5)	20	60	20	0 (4)	100 (5)	80 (5)
Chlorophyta	(42)	7	55	10	5 (41)	7 (42)	36 (42)
Phaeophyta	(19)	0	37	0	0 (18)	25 (19)	50 (19)
Rhodophyta	(43)	10	35	7	0	17 (42)	43 (42)
Tracheophyta	(3)	0	0	0	0	33 (3)	0 (3)

[a] *E.c.* = *Escherichia coli*, *B.s.* = *Bacillus subtilis*, *S.c.* = *Saccharomyces cerevisiae*, *P.a.* = *Penicillium atrovenetum*

[b] Number of species examined same as overall antimicrobial except as noted.

[c] Inhibiting *Herpes simplex* virus, type 1, at ≤200 μg/disk.

[d] Cytotoxic to monkey kidney cells at ≤200 μg/disk.

From Rinehart et al. 1981*b*

TABLE 5. PRIORITY LIST OF SPECIES FROM AHCE 1978 ACTIVE AGAINST *E. COLI*

Shipboard zone of inhibition (mm)[a]	AHCE sample#	Phylum	Secondary testing[b]
31	137	Porifera	–
26	492	Cnidaria	–
25	522	Cnidaria	–
22	547	Chordata	–
21	141	Porifera	NT[c]
20	443	Chordata	–
19	61	Porifera	–
18	92	Porifera	–
	650	Porifera	+
	220	Cnidaria	NT[d]
	755	Chordata	–
	360	Porifera	–
17	64	Porifera	
	292	Porifera	–
	372	Porifera	–
	552	Porifera	–
	399	Porifera	–
	1,237	Rhodophyta	–

[a] From 100 μl of a 20-ml methanol-toluene (3:1) extract of 2 g of sample.

[b] Upjohn screen; + = active against one or more microorganisms at 1,000 μg/ml, dip-spotted.

[c] NT = not tested

[d] Retesting at the University of Illinois indicated no activity versus *E. coli* or *B. subtilis*

From Rinehart et al. 1981*b*

ANTI-INFLAMMATORY SCREENING

Most anti-inflammatory screens involve skin tests on mice. An example is the phorbol myristate acetate-induced mouse ear inflammation (Van Arman 1974).

NEUROTOXICITY SCREENING

Although it is less directly related to the search for drugs *per se*, a screening assay for neurotoxicity nevertheless provides the potential for discovery of neuroregulatory compounds as well as a means of identifying materials of use as neurological probes. Neurotoxicity assays and their relevance are discussed elsewhere in this volume (i.e., Kem, Greenberg and Price, Taylor et al.). A screen employed regularly on extracts on board the R/V *Alpha Helix* (AHBE 1974, AHCE 1978) was an acetylcholine release assay (W. O. McClure, University of Southern California, unpublished observations). Although this assay normally employs mouse brain synaptosomes, it worked equally well on shipboard with readily available fish brain synaptosomes. This assay gives results quickly, but is not as adaptable to mass screening as are the antimicrobial, antiviral, and cytotoxicity assays.

OTHER PHARMACOLOGICAL SCREENING

Marine extracts do have other bioactivities and these have been reported on a regular basis (Fuhrman 1981; Kaul 1983), but none seems likely to lead to a useful drug in the near future.

RESULTS OF SCREENING

DISTRIBUTION OF ACTIVITY

Since the goal of screening is the identification of new pharmaceutical agents, one can fairly ask what activities have been observed thus far and whether any conclusions can be drawn with respect to the incidence of activity by phyla, water temperature, depth, geography, etc.

With respect to phyla, the immediate conclusion is that activity in general is widely distributed, but that specific activities are concentrated in certain phyla, for example, antifungal activity in the holothurins (Echinodermata). During AHCE 1978, about 650 species were examined for antibacterial, antifungal, and antiviral activities, as well as for cytotoxicity. The results are recorded in Table 4. With high incidences of activity in so many phyla, criteria other than activity *per se* (positive-negative reactions) must be employed in deciding which species to investigate more carefully. For that purpose, a semi-quantitative comparison is valuable, such as that of activity versus Gram-negative bacteria as shown in Table 5.

For distribution of activity by depth, the most comprehensive comparisons are those derived from the R/V *Seward Johnson* SeaPharm expeditions to the Western Caribbean in 1985 (Thompson et al. 1986) and to the Galapagos Islands in 1986, summarized in Table 6. While the results for the two areas differ slightly in detail, they reveal similar patterns. Specifically, activity is generally distributed relatively evenly by depth among benthic organisms, at least down to 762 m. Thus, to the extent that different species are found at different depths, screening is well worth while on organisms collected at depths reachable only by a submersible or remotely operated vehicle (ROV), as well as on organisms from shallow water explorable by SCUBA, snorkeling, or shore techniques. The principal deterrent to such widespread screening at greater depths is, of course, cost.

Among the minor variations with depth, antibacterial and antifungal activities are generally reduced in organisms collected in deeper water. This phenomenon may well be related to water

TABLE 6. PROFILE OF ACTIVE SAMPLES.[a]

	AV			AT			AB			AF			ID			IS		
Depth, m	Tstd	Act	%	Tstd	Act	%	Tstd	Act	%	Tstd	Act	%	Tstd	Act	%	Tstd	Act	%
Galapagos																		
>600	147	22	15	150	13	9	150	10	7	150	4	3	150	13	9	150	4	3
600–450	74	8	11	78	13	17	78	10	13	78	3	4	78	22	28	78	4	5
450–300	190	24	13	193	24	12	193	16	8	193	5	3	193	22	11	193	1	1
300–150	56	7	13	62	13	21	62	4	6	62	5	8	62	8	13	62	0	0
<150	22	5	23	30	9	30	30	1	3	30	4	13	30	6	20	30	0	0
SCUBA	287	41	14	328	75	23	328	23	7	328	20	6	328	60	18	328	4	1
Snorkel	112	14	11	123	21	17	123	6	5	123	11	9	123	7	6	123	0	0
Total	888	119	13	964	168	17	964	60	6	964	52	5	964	138	14	964	13	1
Cocos																		
>600	49	6	12	49	7	14	49	8	16	49	4	8	49	6	12	49	1	2
600–450	15	1	7	28	2	7	28	4	14	28	0	0	28	4	14	28	0	0
450–300	20	1	5	35	2	6	35	4	11	35	4	11	35	10	29	35	1	3
300–150	14	2	14	15	2	13	15	2	13	15	0	0	15	0	0	15	0	0
<150	13	0	0	16	1	7	16	0	0	16	1	7	16	1	6	16	0	0
SCUBA	38	11	29	50	18	36	50	9	18	50	8	16	50	10	20	50	0	0
Snorkel	11	1	9	12	2	17	12	2	17	12	0	0	12	1	8	12	0	0
Total	160	22	14	205	34	17	205	29	14	205	17	8	205	32	16	205	2	1
Perlas																		
<150	37	9	24	19	14	74	37	9	24	37	1	3	37	10	27	37	1	3
SCUBA/Snorkel	88	18	20	74	24	32	84	18	21	84	8	10	88	21	24	88	0	0
Total	125	27	22	93	38	41	121	27	22	121	9	7	125	31	25	125	1	1
Total	1,173	168	14	1,262	240	19	1,290	116	9	1,290	78	6	1,294	201	16	1,294	16	1

[a] AV = antiviral, AT = antitumor, AB = antibacterial, AF = antifungal, ID = immunodepressant, IS = immunostimulatory.

temperature, since the incidence of antibacterial and antifungal activity in organisms collected from the west coast of Spain as well as in Maine and Nova Scotia (Table 7) is relatively low, while cytotoxicity and antiviral activity are in normal ranges.

A shibboleth with regard to geography (and water temperature) is that organisms collected in tropical oceans have much greater bioactivity than those collected in colder water. The study usually cited dealt specifically with ichthyotoxins from sponges and holothurians (Bakus 1974); an extension to other types of activity or organisms is probably unwarranted. It certainly is true that the cold waters of the northeastern United States and eastern Canada, western Spain, and New Zealand (Table 7) are rich repositories of antitumor, cytotoxic, and antiviral species.

EXAMPLES OF CLINICAL CANDIDATES

Future candidates. Large-scale screening of marine species during the past few years has produced a number of exciting leads toward new pharmaceutically useful agents, and I am confident that a symposium held a few years from now would reveal a panoply of compounds undergoing clinical testing. In the antitumor area, for example, halichondrin B has been shown to have T/C 244 against B16 melanoma (Hirata and Uemura 1986), while one of the ecteinascidins has T/C 240 against L1210 leukemia (Holt 1986). In the anti-inflammatory area, manoalide is a potent anti-inflammatory agent for which testing will be described in chapters by Wheeler et al. and by Mayer and Jacobs (this volume). Another anti-inflammatory compound of interest discovered by Jacobs is pseudopterosin. It should be noted here, however, that although this activity of manoalide was discovered during an extensive screening for anti-inflammatory agents, manoalide was initially isolated from the sponge as an antibacterial compound (de Silva and Scheuer 1980). Thus, in a sense, manoalide is the product of a chemistry-driven program in which a known compound was tested for activity. For the moment, most of the exciting compounds remain in preliminary testing at pharmaceutical companies, and the test results are not generally available. There are, however, only two marine-derived compounds in clinical trials, or on the market. Interestingly, both, like manoalide above, provide examples of serendipity.

Didemnin B. Didemnin B, a cyclic depsipeptide (Rinehart et al. 1981*a*), was scheduled to begin in 1987 Phase II clinical trials sponsored by the NCI (Chun et al. 1986). It has recently been synthesized in our laboratory (Rinehart et al. 1987*a*). The activity of *Tridemnum solidum,* the tunicate from which didemnin B was isolated, was first discovered during systematic ex-

TABLE 7. BIOACTIVITY OBSERVED.

		No. (%) active, preliminary results			
Depth, m	No. of samples	Cytotoxic	Antiviral	Antibacterial	Antifungal
SeaPharm Spanish Expedition, March 1986					
3–30		174/491 (35)	26/266 (10)	14/454 (3)	12/454 (3)
University of Illinois, Maine Collection, July 1985					
0–3	58		16 (28)	0/28 (0)	2/28 (7)
3–30	96		25 (26)	4/46 (9)	4/48 (9)
University of Canterbury, New Zealand Collections, 1982–1985[a]					
3–30	1,533				
30–130	216	259 (28)	146 (16)		
>130	54				

[a] Numbers and percentages approximate.

tensive screening for antiviral activity on board the R/V *Alpha Helix* in 1978 (AHCE 1978). As noted above, antiviral screening also provides a measure of cytotoxicity, and *T. solidum* appears not only near the top of the list of most promising antiviral extracts, but also among the most cytotoxic extracts (Rinehart et al. 1981*b*). Hence, the *Trididemnum* extract was sent to The Upjohn Company for cytotoxicity testing against the L1210 leukemia cell line. Isolation of the didemnins was relatively facile; of the three initially obtained, didemnin B displayed superior activity *in vitro,* as well as pronounced activity *in vivo* (T/C 199 versus P388 leukemia). Moreover, *in vivo* activity was demonstrated against B16 melanoma (Li et al. 1981).

The compound was then submitted to the NCI where the P388 and B16 melanoma activities were confirmed. At the NCI, a compound of unknown structure isolated independently from a tunicate by Weinheimer, now at the University of Houston, had also shown activity (J. D. Douros, NCI, and A. J. Weinheimer, pers. comm.), and the Weinheimer compound was subsequently found to be identical with didemnin B. On the basis of these activities, didemnin B was selected for pre-clinical toxicity studies, then for Phase I clinical trials (F. A. Dorr et al., University of Texas Health Science Center, San Antonio, manuscript submitted), and now for Phase II clinical trials, all sponsored by the NCI (Chun et al. 1986; Marsoni et al. 1987).

As noted above, the antitumor activity of *T. solidum* was discovered incidental to its antiviral activity. Didemnin B is also active *in vivo* against both DNA and RNA viruses (Renis et al. 1981; Canonico et al. 1982; Weed and Stringfellow 1983), but toxicity appears to restrict its use to life-threatening situations.

Once the activity of didemnin B was known and its structure established, scientists at the University of Arizona noted the similarity between its structure and that of the immunosuppressive drug cyclosporin A. Their *in vitro* immunoassays revealed didemnin B to be more potent, though less selective, than cyclosporin A (Montgomery and Zukoski 1985; D. W. Montgomery et al., University of Arizona, Tucson, pers. comm.). Immunoregulatory studies are underway *in vivo* (Montgomery et al. 1987; Russell et al. 1987), and the compound may have promise in this area as well.

Didemnin B has other activities; it inhibits the dermatological response to psoralen (Gschwendt et al. 1987*a*) in much the same way that cyclosporin A does (Geschwendt et al. 1985, 1987*b*). It is therefore a potential agent for the treatment of psoriasis, a disease against which cyclosporin A has shown some effectiveness (Ellis et al. 1986).

Ara-C and ara-A. Ara-C (cytosine arabinoside, Cytarabine) and ara-A (adenine arabinoside, Vidarabine) are in clinical use, the former as an antitumor agent and the latter as an antiviral agent. Neither compound was isolated from a natural source during extensive screening for these bioactivities. In fact, neither was isolated initially from a natural source at all. They do represent, however, significant examples of marine-derived natural products because their structures are based on the unusual nucleosides obtained (again serendipitously) by Bergmann during his extensive studies of the steroids present in Caribbean sponges (Bergmann and Feeney 1951; Bergmann and Burke 1956; Cohen 1966). The related compounds, spongouridine and spongothymidine, which crystallized from solution and were recognized as being non-steroidal, proved to be antiviral agents. There followed sporadic attempts to improve the activities of the compounds by synthesizing analogues. This effort culminated in the introduction of cytosine arabinoside (Cytarabine) as a clinically useful antitumor agent in 1969 (Bodey et al. 1969). Some years later, ara-A (Vidarabine) was approved for limited use as an antiviral agent (Buchanan and Hess 1980).

The major points to note here are 1) the length of the time between the discovery of the original arabino bases and their activity and the introduction of the analogues into clinical use, and 2) the facts that the originally isolated compounds did not represent the optimal activity and that extensive structure-activity studies were needed to establish the best compound. Ongoing studies of unusual nucleosides as antiviral agents have progressed beyond ara-C and ara-A, so that acyclovir (De Clercq and Walker 1984; Dolin 1985; Robins 1986) may be regarded as at least a third generation descendant of the marine natural products.

CONCLUSION

The time is ripe for speculation and one can comfortably predict not only that marine natural products will provide a rich source of biologically active compounds of medicinal importance but, even more importantly, that they will provide models on which to base extensive synthetic programs leading to still more efficacious drugs.

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Marine Chemical Ecology and Natural Products Research

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INTRODUCTION

The study of chemical ecology, or ecological biochemistry, is concerned with the function of biochemicals in the ecology and behavior of plant and animal interactions. Studies of chemical ecology in terrestrial habitats have yielded valuable biological and ecological information about plant–herbivore and other predator–prey interactions. Natural products isolated from terrestrial plants and insects, and the biological information about their functions and specificities have provided a foundation for both the pharmaceutical and agrichemical industries. Marine organisms provide an untapped resource for these future biotechnological applications. Several thousand marine natural products have been chemically defined; many of these are biologically active compounds possessing novel functional groups and molecular structures. In contrast to terrestrial studies, little is known about the natural functions of these metabolites in the marine environment. Investigations of the role of these metabolites as antimicrobial, antifouling, and/or predator deterrent agents will provide a rationale for developing future applications for these compounds.

In this paper, I review current research in the field of marine chemical ecology and discuss the potential contribution of chemical ecology to biomedical research. Chemical defenses in marine organisms have been proposed to play a critical role in the behavioral and ecological interactions of predators and their prey in marine communities, with little experimental evidence to support these hypotheses. It is critical to use natural herbivores and predators in relevant laboratory and field experiments to test these hypotheses. Increasing our knowledge of the ecological functions of marine natural products will provide information on their specificities and mechanisms of action. Thus we gain insight into the chemical basis of ecologically significant interactions among marine organisms, which can then be applied to collecting and screening marine organisms for other biological and biomedical activities.

DEVELOPMENT OF THE FIELD OF CHEMICAL ECOLOGY

The field of chemical ecology has developed within the last few decades primarily as a result of research in terrestrial natural products chemistry and the biology of plant–herbivore, plant–plant, and predator–prey interactions. Chemists have realized that the molecules they isolate and structurally define often have potent biological activities and have likely evolved for specific biological functions. Biologists and ecologists have realized that chemical substances, particularly the secondary metabolites such as alkaloids, terpenoids, acetogenins, and aromatics, play an important role in the complex behavioral and ecological interactions among organisms. The field of chemical ecology is interdisciplinary in scope, and research has advanced most rapidly as a result of collaboration among chemists and biologists.

Over 12,000 natural products have been described from terrestrial plants and insects (Devon and Scott 1972). Many of these compounds, and biological and pharmacological information about their specificities and functions, have provided a foundation for both the pharmaceutical and agrichemical industries. For instance, knowledge of plant–insect interactions mediated by defensive compounds and pheromones has led to applications in (1) control of insect pests and microbial diseases in crop plants, and (2) conservation of natural communities. Much of the pharmaceutical industry is based on terrestrial natural products or compounds modeled after these natural products.

Terrestrial research in chemical ecology has also yielded information about the ecology, evolution, and coevolution of plants and animals. Numerous articles and books review the topic of chemical ecology and propose hypotheses about the evolution of chemical defenses in plants and animals (e.g., Feeny 1976; Rhoades and Cates 1976; Harborne 1977, 1978; Rosenthal and Janzen 1979; Fox 1981; Crawley 1983; Denno and McClure 1983). These studies suggest that the evolution of plant defense mechanisms is responsive to the plant's risk of discovery by herbivores, the cost of defense, and the relative value of various plant parts (Rhoades 1979). Although there is currently general acceptance of the defensive roles of these compounds, there is considerable speculation regarding how herbivores and the physical environment interact to affect plant chemistry (Coley 1983; Coley et al. 1985; Rhoades 1985). The diversity and ubiquity of the secondary metabolites produced by plants has generated debate regarding their costs and benefits and the selective forces influencing their biosynthesis.

Although thousands of secondary metabolites have now been chemically described from marine seaweeds and invertebrates (Scheuer 1978–83; Faulkner 1984*a*, *b*, 1986), few studies have assessed the ecological roles of these compounds. This is an opportune time for studies in marine chemical ecology since: (1) a strong chemical basis exists regarding the natural products chemistry of marine organisms; (2) our understanding of the complexities of marine communities is advancing rapidly and this facilitates investigations of how chemical interactions affect population and community structure; and (3) marine chemists and biologists are becoming interested in collaborative studies concerning the chemical ecology of marine organisms.

CURRENT RESEARCH IN MARINE CHEMICAL ECOLOGY

The field of marine natural products chemistry has contributed greatly to our understanding of marine chemical ecology. Many chemists have been interested in the biological functions of the compounds they isolate, for both ecological and biomedical research. The literature in marine chemical ecology is scattered throughout the biological and chemical literature, but has been recently reviewed (Bakus et al. 1986). Topics of interest in chemical ecology include symbiosis and mutualism, chemore-

ception, chemical communication, microbial interactions, antipredation, and antifouling. Secondary metabolites from marine organisms have been hypothesized to function as toxins, feeding deterrents, antifouling agents, antimicrobial agents, settling cues, and compounds that mediate competitive interactions between organisms. Little experimental evidence exists to support these proposals, but some recent research will be reviewed here. This manuscript is not intended to be a comprehensive review of marine chemical ecology, but will review selected research that integrates natural products chemistry and marine ecology.

Antipredation

Predators (including herbivores) may exert strong selective pressures on prey organisms. Chemical defenses and protective morphology (tough or calcified textures) have been proposed as important adaptations against predators. In coral reef habitats where herbivory is intense (Carpenter 1986; Lewis 1986), many species of seaweeds produce bioactive secondary metabolites. These compounds have been hypothesized to play a role in chemical defense (Norris and Fenical 1982; Hay 1984; Paul and Hay 1986).

Laboratory and field experiments are now being used to examine the feeding deterrent effects of algal natural products. Several compounds from green seaweeds, including halimedatetraacetate, halimedatrial, and caulerpenyne, have been found to be feeding deterrents toward potential herbivores in laboratory assays (Paul and Fenical 1986; Targett et al. 1986). Field assays have also been developed to examine the feeding deterrent effects of algal extracts and isolated secondary metabolites toward natural populations of herbivores on coral reefs (Paul 1987; Hay et al. 1987*b*; Paul et al. 1987; Paul and Van Alstyne 1988). All of these investigations have shown that some algal metabolites are feeding deterrents and others show no deterrent effects toward particular herbivores. In addition, different species of herbivores respond differently to algal metabolites. Algal secondary metabolites that deter fishes may have no effect on herbivorous amphipods or sea urchins (Hay et al. 1987*a*, *b*; Paul et al. 1987).

Temperate species of brown algae (kelps) have also been shown to be chemically defended against invertebrate herbivores such as the littorine snails and sea urchins (Geiselman and McConnell 1981; Steinberg 1984, 1985). These algae produce polyphenolics that are structurally related to the terrestrial polyphenolics and tannins. Polyphenolics are not found in high concentrations in related species of tropical brown algae (Steinberg 1986).

Many species of sessile marine invertebrates produce bioactive natural products that have been proposed as chemical defenses. I am currently testing the feeding deterrent role of sponge and tunicate compounds in laboratory and field assays on Guam. As with the seaweed chemical defenses, I observe that some compounds are deterrents and others are not, and that different species of predators may respond differently to chemical deterrents. Thompson et al. (1985) found that many temperate sponge compounds were feeding deterrents toward fishes. Some metabolites from marine molluscs such as nudibranchs and the limpet *Collisella limatula* have also been found to be fish feeding deterrents (Thompson et al. 1982; Pawlik et al. 1986).

Soft corals (Alcyonacea) and gorgonian corals (Gorgonacea) also produce a variety of secondary metabolites (generally terpenoids) that are presumed to function in chemical defense. These organisms have few natural predators except for some species of butterflyfishes (Chaetodontidae) (Anderson et al. 1981; Lasker 1985) and some molluscs (Gerhart 1986). Many soft corals have been shown to contain toxins that may function in predator defense (Coll et al. 1982*a*; La Barre et al. 1986*a*). The egg cowry *Ovula ovum* feeds on *Sarcophyton* sp. and converts the major secondary metabolite of the soft coral, sarcophytoxide, to a less toxic compound (Coll et al. 1983). This appears to be a detoxification mechanism that occurs in the digestive gland of the cowry. The cowry *Cyphoma gibbosum* feeds on species of gorgonians in the Caribbean (Birkeland and Gregory 1975; Gerhart 1986; Harvell and Suchanek 1987). Prostaglandin A2, a secondary metabolite of *Plexaura homomalla,* has been shown to function as a feeding deterrent toward wrasses in field assays (Gerhart 1984).

Many species of opisthobranch molluscs are specialized predators on organisms that produce secondary metabolites. These opisthobranchs include: (1) the nudibranchs (order Nudibranchia) that feed on sponges and coelenterates; (2) the sea hares (Anaspidea) that feed on seaweeds; and (3) the ascoglossans (Ascoglossa = Sacoglossa) that feed on siphonous green algae. Most of these animals sequester secondary metabolites from their dietary sources with little or no modification of the chemical structures (Faulkner and Ghiselin 1983; Faulkner 1984*a*, *b*, 1986). These compounds are stored in glands, are exuded by the opisthobranchs when attacked or molested, and are presumed to function as chemical defenses. However, experiments critically testing these hypotheses and the defensive roles of these compounds toward predators have rarely been conducted (Thompson et al. 1982).

Research in the field of predator and herbivore deterrents is just beginning. Many questions regarding the costs and benefits of secondary metabolite production, chemical variation, mechanisms of detoxification, and coevolution of specialist predators and their prey remain. It is critical that experiments be designed that use natural predators to examine the deterrent effects and ecological importance of these compounds.

Competition for Settling Space

Secondary metabolites have been implicated in mediating competitive interactions for settling space in marine habitats, especially on coral reefs where space may limit recruitment (Jackson and Buss 1975). Little experimental evidence currently exists to support this hypothesis. Soft corals actively exude secondary metabolites that show allelopathic effects toward scleractinian corals (Coll et al. 1982*b*; La Barre and Coll 1982; Sammarco et al. 1983, 1985; La Barre et al. 1986*b*). The temperate sponge *Aplysina fistularis* has also been shown to exude metabolites that may defend against settling organisms, fouling organisms, and predators (Thompson 1985; Walker et al. 1985). The sponge *Siphonodictyon coralliphagum* produces the metabolite siphonodictine that is toxic to the coral *Acropora formosa* (see Sullivan et al. 1983).

Settling Cues and Antifouling

Larval settling behavior is responsive to many factors including substratum type, microbial surface films, and light in-

tensity. Morse et al. (1979, 1980, 1984) have shown that gamma-aminobutyric acid and macromolecules from cyanobacteria and red algae can induce the settlement of the abalone *Haliotis*. Pawlik (1986) showed that specific free fatty acids induced settling and metamorphosis of the sabellarid polychaete *Phragmatopoma californica*.

It is hypothesized that secondary metabolites function as antifouling agents in seaweeds and marine invertebrates. Many sessile invertebrates such as holothurians, sponges, ascidians, soft corals, and gorgonians have clean surfaces, suggesting that secondary metabolites present in these organisms may function in antifouling. Only a few of these organisms have been demonstrated to exude metabolites into seawater, a process that should be necessary to prevent the settling of fouling organisms (Coll et al. 1982*b*; Thompson 1985; Walker et al. 1985). Temperate sponges have been shown to possess antifouling activity toward algae and invertebrates (Thompson 1985; Thompson et al. 1985). Targett et al. (1983) showed that homarine from gorgonians could inhibit the growth of the diatom *Navicula salinicola*. The muricins, aminogalactose saponins from the gorgonian *Muricea fructicosa*, inhibited the growth of the diatom *Phaeodactylum tricornutum* (see Bandurraga and Fenical 1985). Some temperate seaweeds, including *Sargassum* and *Rhodomela*, exude polyphenolics and bromophenols that may have a role in antifouling (Sieburth and Conover 1965; McLachlan and Craigie 1966; Al-Ogily and Knight-Jones 1977; Phillips and Towers 1982).

Symbiosis and Mutualism

Fascinating examples of symbiosis exist in the marine environment; the sea anemone–clownfish interaction is a well-known example. Some clownfishes inhabit many or most of the 10 known species of host anemones, whereas others are specific to one; reciprocally, the anemones associate with from one to 11 fishes (Dunn 1981). Small water-soluble molecules—amphikuemin, tyramine, and tryptamine—produced by the anemones mediate the species-specific attraction of the clownfishes (Murata et al. 1986; Nakanishi, this volume).

Increased understanding of symbiosis between tropical marine invertebrates and associated microorganisms could lead to a better understanding of the biosynthesis of marine natural products. For many marine organisms, it is unclear whether the secondary metabolites they contain are produced by the host, by the symbiotic microorganism, or by some combination of both (Dunn et al. 1975; Kokke et al. 1981). Additionally, the mechanisms involved in "organelle symbiosis" (exhibited by some opisthobranchs such as the ascoglossans that sequester functioning chloroplasts from their algal food [Trench 1975, 1980] and some aeolid nudibranchs that sequester functioning nematocysts from coelenterates [Edmunds 1966; Mariscal 1974]) warrant further study. Many issues exist concerning symbiotic associations in marine organisms including: (1) factors mediating host–symbiont tissue compatibility; (2) other chemical cues that induce or mediate symbiotic associations; and (3) factors influencing settlement of the symbiont and host. Research into these and other basic questions will yield information of biological and biomedical importance. For example, studies of reef corals and their associated zooxanthellae (dinoflagellates) have already contributed to knowledge of immunological responses, cellular specificity, bioenergetics, and calcification processes (Fitt 1984, 1985*a*, *b*; Barnes 1985; Blank and Trench 1985).

MARINE CHEMICAL ECOLOGY AND BIOMEDICAL RESEARCH

Biomedical applications of natural products from terrestrial sources are widespread and of commercial importance. Much of the world's pharmaceutical industry is based on terrestrial natural products or compounds modeled after natural products. Thus, hundreds of unique, biologically-active natural products isolated from marine organisms provide an untapped resource for future biomedical applications. Understanding the natural functions and specificities of these compounds could provide a rational approach for screening and developing future applications of the compounds. By screening marine organisms for natural products that deter predators or competitors, discoveries and advances in the field of marine natural products could be accelerated. To date, most of the applications for marine natural products, and many of the compounds themselves, have been discovered by large-scale collecting and screening programs. These methods are costly and inefficient since collection of the organisms is generally not based on any biological rationale, and many inactive organisms are collected and screened. Also, some active compounds are likely degraded while the organisms are collected, stored, or extracted. Better knowledge of the natural functions, physiological effects, specificities, mechanisms of action, and detoxification of marine natural products would enable natural products chemists and pharmacologists to focus their efforts on organisms that offer the greatest potential for new discoveries.

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Feeding Deterrents in Molluscs

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INTRODUCTION

There are seven classes of living molluscs: Gastropoda (snails, slugs), Bivalvia (clams, mussels, oysters), Polyplacophora (chitons), Cephalopoda (octopuses, squids), Scaphopoda (tusk shells), Aplacophora, and Monoplacophora. Although the bivalves are of greatest commercial value, the chemist has found that the gastropods contain an unusual array of marine natural products. In particular, those gastropod molluscs, such as sea hares and dorid nudibranchs, that lack the usual molluscan shell often contain toxic or repugnant metabolites from dietary sources.

The earliest research on natural products from marine molluscs was concerned with the ancient pigment "Tyrian purple." These studies have been reviewed by Baker (1974). Studies on the defensive chemicals of molluscs had their origins in reports of the toxicity of sea hares (see Baslow 1969) and the presence of repellent secretions in nudibranchs (Thompson 1960*a*). The first chemical studies of sea hares revealed the presence of brominated terpenes that are strikingly similar to algal metabolites that were described at about the same time. Chemical studies of nudibranchs and pulmonates appeared later, when modern instruments made it feasible to study the small quantities of compounds obtained from these sources. Almost from the very beginning of chemical studies on sea hares and nudibranchs, it was assumed that the chemicals obtained were responsible for the biologists' observations that the shell-less marine molluscs had few predators. It seemed so obvious to chemists that exudation of a toxic or evil-tasting chemical would cause a potential predator to avoid the chemically protected organism that they neglected to demonstrate the efficacy of the pure compounds in ecologically relevant bioassays. The necessity for such bioassays has now been recognized and, fortunately, the data now being accumulated provide considerable support for the hypothesis that shell-less marine molluscs are chemically defended. A selection of metabolites from marine molluscs and their ecological significance will be reviewed.

SEA HARES

In 1963, Yamamura and Hirata described the isolation of the brominated sesquiterpenes aplysin (**1**), debromoaplysin (**2**), and aplysinol (**3**) from the sea hare *Aplysia kurodai*. This was followed by the structural elucidation of a brominated diterpene aplysin-20 (**4**) from the same source (Matsuda et al. 1967). The sesquiterpenes **1** and **2** were soon shown to be the acid-catalyzed cyclization products of laurinterol (**5**) and debromolaurinterol (**6**), metabolites of the red alga *Laurencia okamurai* (see Suzuki et al. 1969). By feeding tritium-labelled laurinterol (**5**) to *Aplysia californica*, it was demonstrated that the same "acid-catalyzed" reaction occurred in the digestive gland of the sea hare (Stallard and Faulkner 1974*b*). Because the same halogenated chemicals were found in the skin and the digestive gland of *Aplysia californica*, we have proposed that halogenated compounds are first stored in the digestive gland then transmitted to the skin where they are released into a distasteful mucoid secretion.

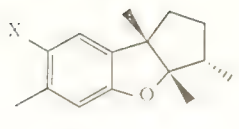
The majority of sea hares contain metabolites obtained from their diets. The Hawaiian sea hare *Stylocheilus longicauda* was shown to contain aplysiatoxin (**7**) (Kato and Scheuer 1974), a toxic compound later traced to the cyanophyte *Lyngbya majuscula* (see Moore et al. 1984). In addition to its toxicity, aplysiatoxin (**7**) was also reported to be an irritant, a property that is probably more relevant than toxicity to its possible role as a chemical deterrent.

Most *Aplysia* species contain halogenated compounds from red algae of the genera *Laurencia* or *Plocamium* (see Faulkner 1984*a*). Halogenated monoterpenes from *A. californica*, exemplified by compounds **8** and **9**, were traced to *Plocamium cartilagineum* and *P. violaceum*, respectively (Faulkner et al. 1973; Stallard and Faulkner 1974*a*; Mynderse and Faulkner 1978). Halogenated lipids of the "C_{15}-enyne" class, exemplified by the ethers **10** and **11** from *Aplysia brasiliana* (see Kinnel et al. 1979) are commonly found in *Aplysia* species: these compounds are typical metabolites of red algae of the genus *Laurencia* (see Faulkner 1984*a*). *Laurencia* species also provide the halogenated sesquiterpenes and diterpenes found in several species of *Aplysia*. For example, *Aplysia dactylomela* from Puerto Rico contains parguerol (**12**) and isoparguerol (**13**) that are both novel diterpenes (Schmitz et al. 1982). A parguerol derivative has been isolated from *Laurencia obtusa* collected in Britain (Higgs and Faulkner 1982).

Several sea hares, particularly those of the genus *Dolabella*, contain diterpenes that can be obtained from brown algae on which the animals graze. A novel class of diterpenes known as the dolabellanes (e.g., **14**) were isolated first from *D. californica* (see Ireland and Faulkner 1977) and subsequently from the brown algae *Glossophora galapagensis* (see Sun and Fenical 1979) and *Dictyota dichotoma* (see Amico et al. 1980; Rao et al. 1986). Metabolites of brown algae have also been isolated from *Aplysia vaccaria* (see Midland et al. 1983).

One of the most striking omissions in the chemical studies of sea hares is the lack of data on the biological properties of metabolites isolated from sea hares. Some algal metabolites such as laurinterol (**5**) were reported to possess antimicrobial activity (Sims et al. 1975) yet aplysin (**1**), to which laurinterol (**5**) is converted in the digestive gland of the sea hare, is almost devoid of antimicrobial activity. However, the property of antimicrobial activity may not be relevant to chemical defense. It is thought that the most important biological activity for a defensive metabolite is the ability of the compound to deter potential predators, primarily by taste. The only sea hare metabolites that have the demonstrated ability to inhibit fish feeding are brasilenyne (**10**) and *cis*-dihydrorhodophytin (**11**), both isolated from *A. brasiliana* (see Kinnel et al. 1979). No other studies of feeding inhibition due to sea hare metabolites have been reported.

Despite the fact that sea hares are the largest and the chemically most studied of the opisthobranch molluscs, many details



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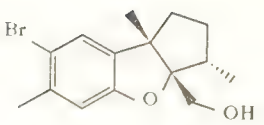

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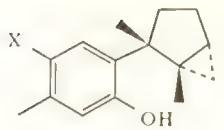

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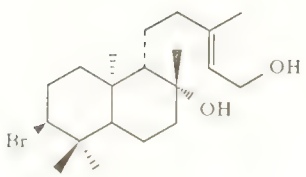

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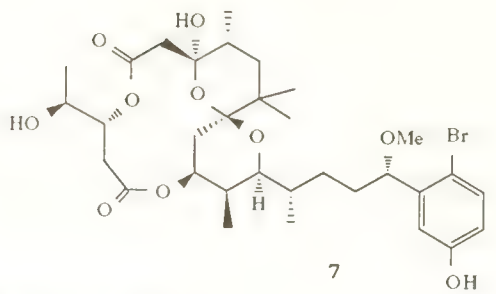

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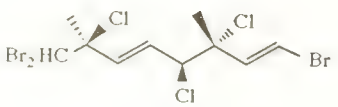

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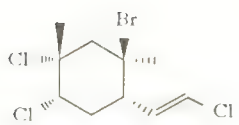

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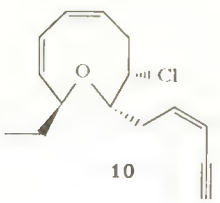

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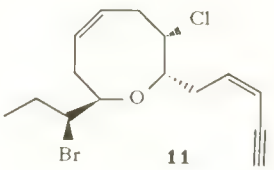

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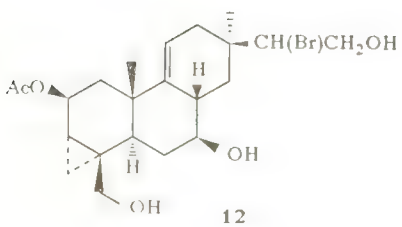

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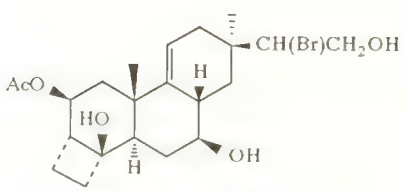

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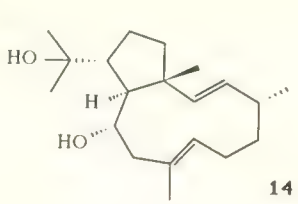
of their chemical defense strategy remain to be demonstrated conclusively. The chemical constituents of secretions from the "ink-gland" (Bochmann's gland) and the opaline gland have not been fully characterized, and their proposed value as defensive metabolites has yet to be determined. It is not known if *all* of the metabolites stored in the digestive gland are eventually transmitted to the skin and utilized in the mucous secretion or whether some compounds are detoxified. There is some largely circumstantial evidence that the eggmasses are chemically protected against predation but the chemicals involved have not been identified.

DORID NUDIBRANCHS

Research on dorid nudibranchs has resulted in an increasingly detailed knowledge of the defensive role of chemicals. The majority of metabolites isolated from these nudibranchs are of dietary origin, generally from sponges or bryozoans. The metabolites are usually stored in cells in the dorsal mantle that were first described by Thompson (1960*b*). When molested, the dorid nudibranch can retract its rhinophores and gill tissue and exude defensive chemicals over the dorsal mantle with the result that only the distasteful mantle tissue is exposed. Repeated molestation of a nudibranch that is kept without the appropriate food results in loss of defensive capability.

The first chemical studies of a nudibranch led to the isolation of an isonitrile, 9-isocyanopupukaenane (**15**), from the mucus secreted by *Phyllidia varicosa* and from the sponge *Hymeniacidon* sp. (see Hagadone et al. 1979). This chemical investigation extended the earlier research by Johannes (1963) who had reported that the mucus contained a volatile, tasteless (!), strong-smelling toxin. The serendipitous identification of a sponge as the source of 9-isocyanopupukaenane (**15**) allowed accumulation of sufficient material for structural elucidation. Unfortunately, no bioassays were performed to show that **15** was the



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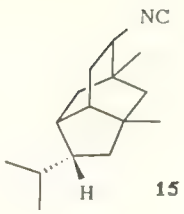

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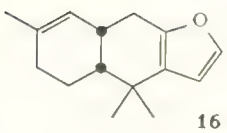

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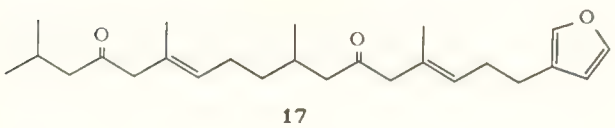

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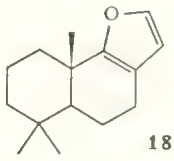

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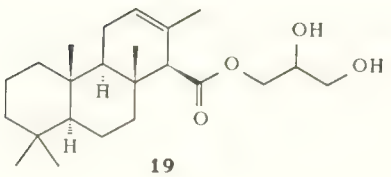

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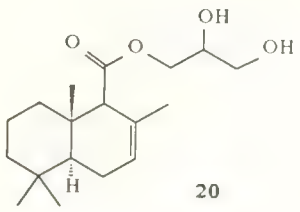

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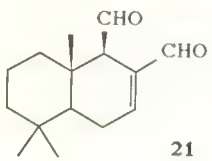

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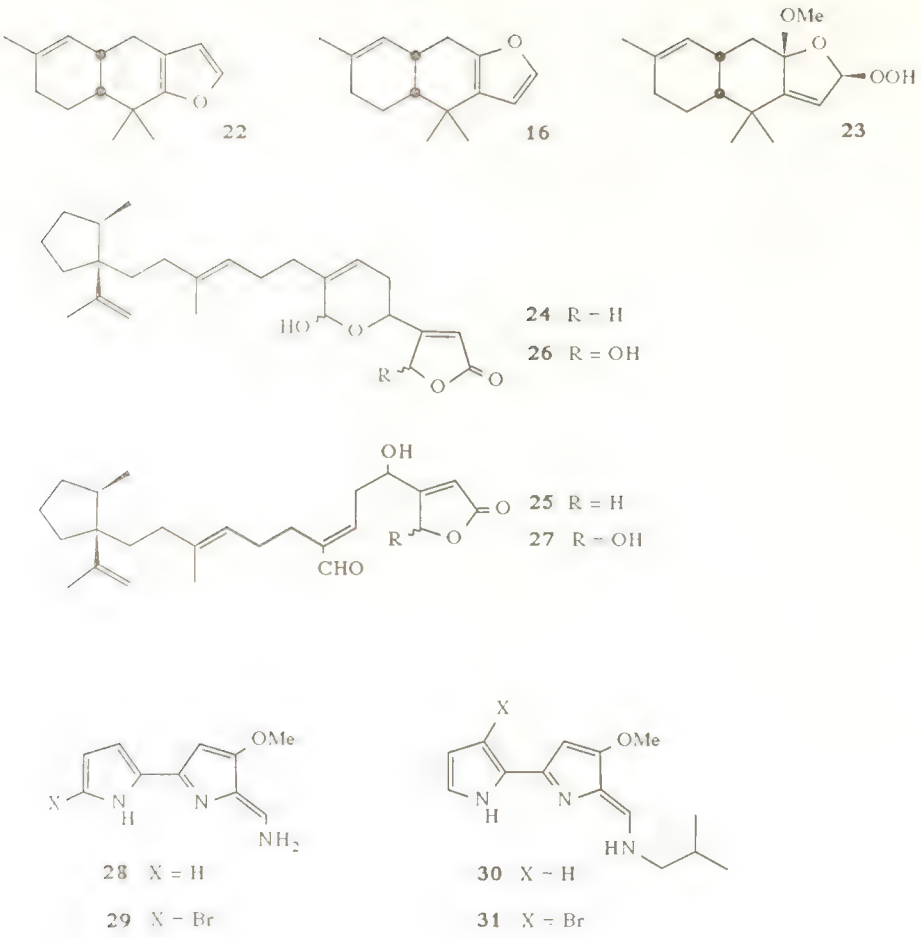
active compound, presumably because the circumstantial evidence was compelling.

With recent advances in instrumentation, it is possible to identify the metabolites from collections of nudibranchs and occasionally from individuals, although in order to study the biological properties of the compounds isolated from nudibranchs it is still advantageous to find a dietary source. The nudibranch *Cadlina luteomarginata* contains two types of sponge metabolites. A group of isonitriles and related molecules were traced to a species of *Axinella*, and a number of furans from sources such as *Eurypongia* sp., *Spongia idia*, and *Dysidea amblia* were isolated (Thompson et al. 1982). The metabolites were located in ducts on the surface of the dorsal mantle. The nudibranchs contained only a selected group of sponge metabolites although they were known to feed on many sponge species. The mechanism by which nudibranchs differentiate between sponge metabolites is unknown. Furodysinin (**16**), idiadione (**17**), pallescensin-A (**18**), a mixture of sesquiterpene isonitriles, and a mixture of the corresponding isothiocyanates were all ichthyotoxic and inhibited fish feeding. Comparison of the metabolites of *C. luteomarginata* from La Jolla (Thompson et al. 1982) with those of British Columbia specimens of the same animals (Hellou et al. 1982) revealed only one common metabolite. This is not surprising because there are considerable differences in the sponge fauna of the two locations. In contrast, the diterpene glyceride **19** and the sesquiterpene glyceride **20** were found in specimens of *Archidoris montereyensis* collected at both locations (Gustafson et al. 1984). It is significant that the glycerides **19** and **20** are synthesized by the nudibranch and are not obtained from a dietary source, despite the fact that both compounds resemble metabolites found in sponges (Gustafson and Andersen 1985). A second example of a compound produced by *de novo* biosynthesis is polygodial (**21**), which was first isolated from *Dendrodoris limbata* (see Cimino et al. 1983; Cimino et al. 1985). Polygodial (**21**) was later found in three other species of *Dendrodoris* from Hawaii and the Gulf of California (Okuda et al. 1983). By comparing all data on nudibranchs common to both La Jolla and Vancouver, Raymond Andersen and I propose that variation of the metabolites of a nudibranch over a broad geographic range is indicative of a dietary source for the compounds while isolation of the same metabolite(s) from a single species or from related species collected over a wide geographical range suggests that the compounds are produced by *de novo* biosynthesis.

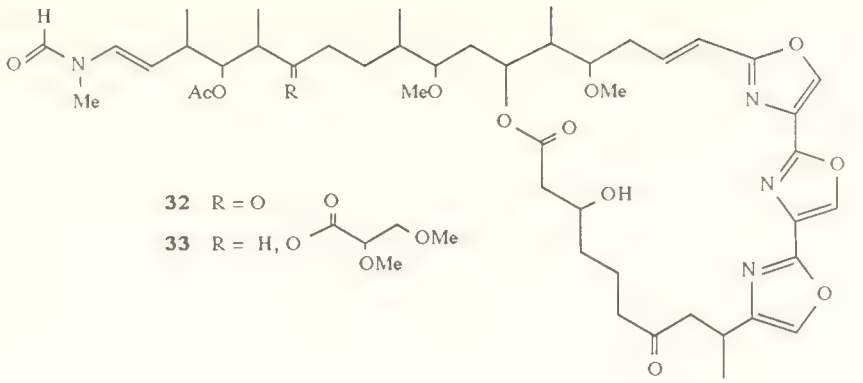
There are many examples of metabolites isolated from nudibranchs that differ slightly from known sponge metabolites. There are two possible reasons for the differences: the trivial rationale is that we have not located the sponge that produced the metabolites, but more interesting is the possibility that the nudibranch has modified metabolites obtained from a sponge.

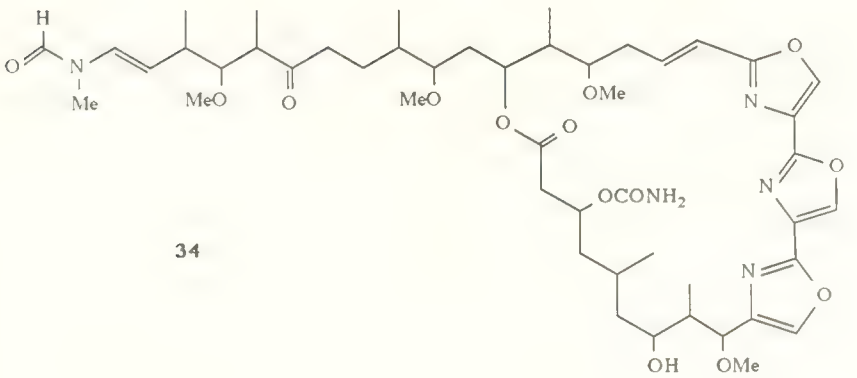


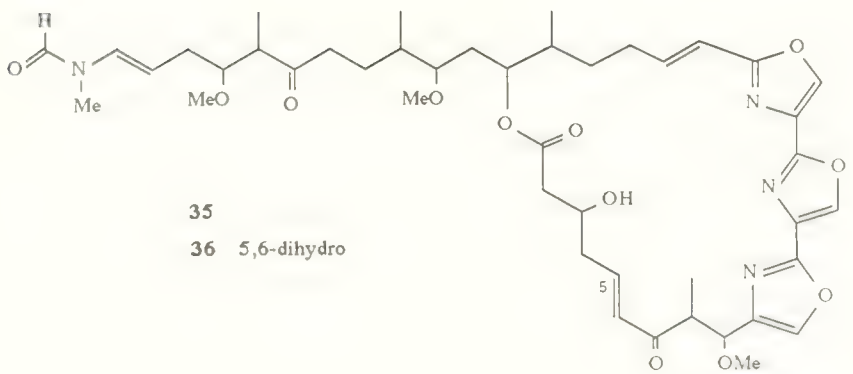
Recent research on the Palauan nudibranch *Chromodoris funerea* provided good evidence that the nudibranch oxidizes furodysin (**22**) and furodysinin (**16**) to produce relatively unstable products that are the same as those produced by singlet oxygen oxidation of the furan (Carté et al. 1986). The most interesting of these products is furodysin hydroperoxide (**23**), which may result from trapping of an endoperoxide by the extraction solvent methanol. Examination of a population of *C. funerea* collected in a marine lake adjacent to the original collection site resulted in the isolation of a completely different set of compounds. The marine lake contains a very different sponge fauna, related to its shaded location, and the nudibranchs contain luffariellins C (**24**) and D (**25**), which are reduction products of the known sponge metabolites luffariellins A (**26**) and B (**27**) (Kernan et al. 1987). It appears that *C. funerea* is quite adaptable when confronted with the task of acquiring defensive chemicals from dietary sources.

Nudibranchs have few known predators, most of which are other opisthobranchs. Just as nudibranchs can tolerate the distasteful metabolites produced by sponges, carnivorous molluscs can tolerate the same compounds that comprise the nudibranch's defensive arsenal, unless the compounds are too highly concentrated. The nembrothid nudibranch *Tambje abdere* can exude sufficient quantity of a mixture of tambjamines (**28–31**) to deter most attacks by its carnivorous relative *Roboastra tigris* (see Carté and Faulkner 1986). The related nudibranch *T. eliora* cannot secrete a sufficient quantity of the tambjamines to deter *R. tigris* and therefore attempts to escape by swimming. Both *T. abdere* and *T. eliora* obtain the tambjamines (**28–31**) from a dietary source, the bryozoan *Sessibugula translucens* (see Carté and Faulkner 1983). In a Y-maze experiment, *T. eliora* was attracted to low concentrations of tambjamines but was repelled by seawater containing higher concentrations of the same mixture (Carté and Faulkner 1986). These data suggest that *T. eliora* may locate its food by detecting low concentrations of the tambjamines but may perceive higher concentrations as a danger signal.

Since the feeding inhibitors from nudibranchs act on the gustatory tissues of fish at very low concentrations, it might be reasonable to expect that some of these compounds will show activity in unrelated pharmacological assays. Relatively few compounds from nudibranchs have received adequate pharmacological screening due to the small quantities available, but some interesting data have been accumulated for the few examples that have been screened. In 1986, Roesener and Scheuer reported the isolation of two macrolides, ulapualides-A (**32**) and -B (**33**), in the eggmasses of *Hexabranchus sanguineus*. Simultaneously, Matsunaga et al. (1986) described the isolation of kabiramide C (**34**) from eggmasses that could reasonably be attributed to *H. sanguineus*. We have found both kabiramide C (**34**) and a new macrolide halichondramide (**35**) in two specimens of *Halichondria* sp. from Palau and Kwajelein, respec-







tively (Kernan and Faulkner 1987). It seemed obvious that related compounds should be found in the nudibranch, yet Roesener and Scheuer reported only very low concentrations in the animals. We have therefore studied these compounds and their roles in the sponge-nudibranch food chain in some detail.

Hexabranchus sanguineus strongly prefers the sponge *Halichondria* sp. It ate freeze-dried specimens of *Halichondria* but refused to eat freshly collected temperate sponges. We have so far isolated a total of nine related macrolides from specimens of *H. sanguineus* and *Halichondria* sp. The macrolides are found in relatively high concentrations in the secretion of *H. sanguineus* and are also found in the mantle and the digestive gland, which includes the gonad, but are not found in the accessory reproductive organs. The only compound that we have found in both sponge and nudibranch is 5,6-dihydrohalichondramide (**36**): in specimens from Kwajelein, halichondramide (**35**) was the major sponge metabolite while the dihydro derivative **36** was dominant in the nudibranch. Both kabiramide C (**34**) and halichondramide (**35**) are very effective fish-feeding inhibitors and exhibit an ED_{50} of 0.1 μg/mg food pellet against *Thalassoma lunare*, a carnivorous fish commonly found in the Indo-Pacific region. All of the trisoxazole macrolides have pronounced antifungal activity, comparable to that of commercially available antifungal agents. The ulapualides (**32** and **33**) inhibit L1210 leukemia cell proliferation and kabiramide C (**34**) inhibits PMA-induced inflammation in the mouse ear. Unfortunately, all of these compounds may be too toxic for internal use (mouse toxicity by subcutaneous injection at ca. 1 mg/kg), but topical use is not completely excluded.

THE EVOLUTION OF THE CHEMICAL DEFENSE MECHANISM

During the evolution of the opisthobranch molluscs there has been a general trend toward loss of the shell. Sea slugs have evolved from marine snails. Several adaptations have occurred that compensate for the loss of the physical protection afforded by a shell. Eolid nudibranchs use nematocysts acquired from coelenterates on which they prey for their own protection. Sea hares, sacoglossans, dorid nudibranchs, and some pulmonates can use dietary-derived chemicals to deter predators.

Faulkner and Ghiselin (1983) have proposed that the evolution of a defense mechanism based on dietary chemicals has rendered the physical protection afforded by a shell obsolete, leading to its gradual loss. In the case of a dorid nudibranch that employs sponge metabolites, the following evolutionary scheme is proposed. The ancestral shelled mollusc became adapted to feeding on sponges. This required that the mollusc deal with both spicules and chemical deterrents that protect the sponge. A gradual change from excreting the chemical deterrents in the feces to storing them in specialized skin glands allowed the mollusc to use the chemical defenses of sponges for its own protection. Only then was the ancestral mollusc able to do without a shell, which was gradually lost. A similar evolutionary scheme is proposed for the sea hare, except that the sea hare derives its deterrents from an algal diet, and has a vestigial shell under the mantle.

The evolutionary advantages of a defensive strategy based on dietary chemicals are that the cost of acquiring the chemicals is minimal while the cost of producing and transporting a shell is saved. One disadvantage of this defensive strategy is that the nudibranch is dependent on finding sponges that contain deterrents in order to maintain its protective secretions. This disadvantage has been overcome by a few species of nudibranchs that are capable of *de novo* synthesis of secondary metabolites. If the loss of the shell is advantageous, the existence of both a shell and chemical deterrents in pulmonates and at least one limpet must be explained. It seems reasonable to propose that, in addition to a chemical defense against predators, intertidal molluscs need a shell to prevent dehydration and physical damage.

PULMONATES AND LIMPETS

The molluscs of the rocky intertidal zone face a particularly harsh environment. At high tide they are battered by waves that often carry stones or debris, and at low tide they face desiccation. They require a shell for protection against these physical rigors but when they forage for food they can derive considerable advantage from a chemical defense.

Marine pulmonates are air-breathing molluscs that live in intertidal zones. Although the majority of marine pulmonates have shells and live on rocks, some species, such as *Onchidella binneyi*, have a leathery skin and hide under rocks or burrow into the substrate at the water's edge. The chemical defense system of *O. binneyi* is among the most obvious and was the first to be studied (Ireland and Faulkner 1978). When molested, *O. binneyi* expels a defensive secretion from apical pores in papillae situated around the edge of the mantle. The secretion, which was collected in capillary tubes, consisted of a single nonpolar ichthyotoxin, onchidal (**37**) in a mucus that serves both to deliver the toxin and hold it in contact with the potential predator. Onchidal (**37**) has also been isolated from *O. borealis* and *O. patelloides*, and is therefore considered to be synthesized by the animals (unpublished data).

Members of the genus *Siphonaria* (false limpets) contain "polypropionate" metabolites, exemplified by diemenensin-A (**38**) from *S. dimenensis* (see Hochlowski and Faulkner 1983) and denticulatin-A (**39**) from *S. denticulata* (see Hochlowski et al. 1983). The polypropionates have been proposed as defensive metabolites but there is little evidence to support the hypothesis. In fact, field experiments on *Siphonaria maura* suggest that the animal is poorly protected against predators when detached from a rock and inverted in a tide pool. Polypropionate metabolites have also been isolated from sacoglossans, and we have determined that one of these metabolites, tridachione (**40**) from *Tridachiella diomedea* (see Ireland and Faulkner 1981), inhibits fish feeding at 5 μg/mg in food pellets.

In contrast with *Siphonaria* spp., the cave-dwelling pulmonate *Trimusculus reticulatus* is able to deter starfish with a mucus secretion. The only nonpolar metabolite in the mucus is a diterpene diol (**41**) (Manker and Faulkner 1987) but attempts to deter starfish using the pure compound have been unsuccessful, perhaps because it has not been possible to recreate both the physical and chemical properties of the defensive secretion.

An investigation of the limpets found along the rocky intertidal coast of southern California revealed that only one of five species is chemically protected. Limatulone (**42**), a symmetrical triterpene related to squalene, is a metabolite of *Collisella limatula* that is among the most effective fish feeding inhibitors (Albizati et al. 1985). Limatulone (**42**) was found to be concentrated in the foot of *C. limatula*. When the shell of *C. limatula* is struck by a rock, it does not smash, but instead the outer rim of the shell breaks off, absorbing the blow, and the foot is exposed (Pawlik et al. 1986). Without the protection afforded by limatulone (**42**), the exposed foot could easily be attacked by predatory fish and crabs.

CONCLUSIONS

A large number of very interesting natural products have been isolated from marine molluscs (Faulkner 1984*a*, *b*, 1986, 1987). The underlying hypothesis that sessile or slow-moving invertebrates that lack physical protection will necessarily have an alternative defensive strategy seems to be supported by the frequency with which biologically-active molecules having unique chemical structures are isolated from nudibranchs and sea hares. However, very few of these molecules have been assayed for their ability to deter predators. There is strong circumstantial evidence and a growing body of experimental data to support the hypothesis that feeding deterrence can be attributed to chemicals isolated from molluscs but more experimental evidence must be accumulated in order to convince ecologists that chemical defense plays a major role in the life histories of shell-less molluscs.

ACKNOWLEDGMENTS

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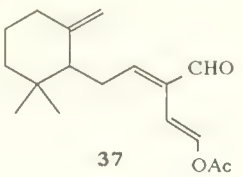

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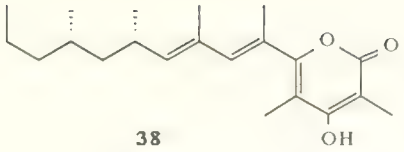

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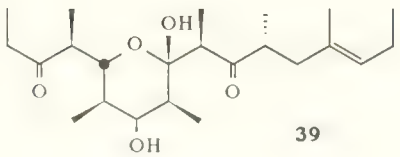

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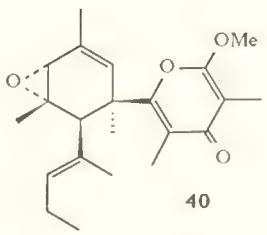

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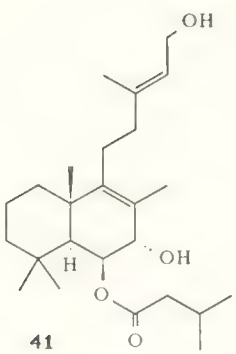

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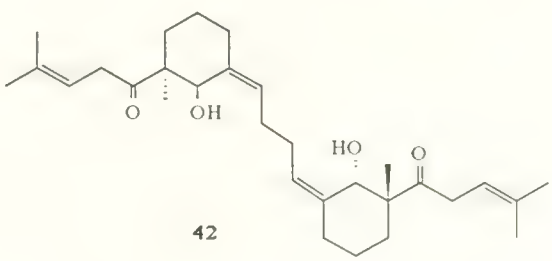

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T. Ghiselin, Mary Kay Harper, Jill E. Hochlowski, Chris Ireland, Robert S. Jacobs, Michael R. Kernan, Denise C. Manker, Tadeusz F. Molinski, Joseph R. Pawlik, Janice E. Thompson, Roger P. Walker and Stephen J. Wratten. Research in my laboratory was supported primarily by grants from the National Science Foundation with additional support and research collaboration from Allergan Pharmaceuticals, Smith, Kline and French, and Syntex Research Laboratories.

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Ethno-Natural Historical Leads

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INTRODUCTION

Terrestrial higher plants furnished virtually all raw material for natural product research from the 19th century on until World War II. Ethno-natural history provided many important leads for the scientist; notable examples include euphoria-inducing opium poppies, the antimalarial cinchona bark, and recently, the sweetener hernandulcin (Compadre et al. 1985). The discovery of the antibiotic properties of penicillin extended natural product research to lower plants, which had a limited ethno-natural history. The huge success and biomedical significance of this facet of natural product research rest on a twin base, the ease with which large numbers of organisms can be sampled and evaluated in a short time, and the ready adaptation of fermentation technology that permits large-scale production.

Marine natural products research has derived leads from many approaches, including a few from ethno-natural history. Not surprisingly, organisms that constitute a public health hazard, e.g., tetrodotoxin and the red tide organisms, are prime examples. There are valid reasons why such leads are sparse. Man's exploration of the oceans lags far behind similar pursuits on land. Equally important is the fact that the major cultures forced to rely on marine resources, notably the small land-poor islands and atolls of Oceania, lacked written languages for most of their history.

The peoples of Asia, principally Chinese and Japanese, are best known for their extensive utilization of the marine flora and fauna. One would expect the accessibility and familiarity are important factors in selecting or discovering medicinal uses of marine biota. Hence it is not surprising that seaweeds, fishes, and molluscs have received greater attention than, say, sponges or tunicates. I have chosen a phyletic rather than a geographic organization in this attempt to compile and perhaps uncover some ethno-natural historical leads for biomedicine based on marine resources.

SEAWEEDS

Chapman's (1950) monograph, *Seaweeds and their Uses* includes, in about 250 pages of text, a five-page section on Seaweeds in Medicine. In his historical introduction, Chapman points out that algae were not highly regarded by the early peoples in the West, which is in sharp contrast to the great esteem in which seaweeds were held in the Orient. Most of the references (Chapman 1950) to medicinal uses of seaweeds in the ancient Chinese *Materia medica* tend to be oblique or are descriptions of multi-component concoctions, not unlike accounts of folk medicines based on terrestrial plants. It is worth noting that the low incidence of goiter in China and Japan is probably due to the extensive use of iodine-rich algae in the diet (Chapman 1950).

Anthelmintics occupied an important niche in many ancient pharmacopeias. Chapman (1950) lists a number of algal species that have been used in various parts of the world as vermifuges. Among them *Alsidium helminthochorton* in Greece and Turkey, and two species of *Chondria, Rhodymenia* sp., and *Durvillea* sp. by the Maoris. Most notable among the anthelmintic algae is *Digenea simplex,* also referred to as "Corsican weed" (Hashimoto 1979). Its active principle, α-kainic acid, was isolated and characterized by Japanese workers in the 1950s. This was followed by the isolation, also in Japan, of a related anthelmintic, domoic acid, from *Chondria armata* (see Scheuer 1973). Nearly twenty years later, it was discovered that α-kainic acid and related amino acids possess highly selective neurobiological properties (McGeer et al. 1978), which render them valuable tools in the study of Huntington's disease and epilepsy. α-Kainic acid acts by killing nerve dendrites but not the axons. It is worth noting that a structural subunit in these compounds is GABA (γ-aminobutyric acid), a substance that inhibits nerve transmission in the mammalian brain. Some of these acids also possess insecticidal activities. New variants of α-kainic acid continue to be isolated (Maeda et al. 1986).

Other algae used in medicine that are mentioned by Chapman (1950) include *Gelidium cartilagineum* and *Dictyopteris polypodiodes,* which have been used against lung diseases and scrofula; *Laminaria saccharina* against syphilis; *Iridophycus flaccidum* and *Delesseria sanguinea* as anti-coagulants; and *Hypnea nidifica* and *Centroceras clavalatum* for stomach troubles.

An expanded listing of algae in folk medicine may be found in Volume 1 of *Marine Algae in Pharmaceutical Science* (Hoppe et al. 1979). The descriptions are brief, but the bibliography appears to be comprehensive. Interestingly, in Volume 2, which was published in 1982 (Hoppe and Levring 1982), emphasis is shifted to chemical and biochemical studies of algal constituents. Folk medicinal uses of algae are no longer mentioned.

A tersely annotated bibliography of algae in medicine compiled by Stein and Borden (1982) may be found in a volume of conference proceedings.

A Chinese publication (South China Sea Institute of Oceanology 1978) of medicinal uses of marine organisms of the South China Sea was brought to my attention and loaned to me by Dr. William Fenical. A first-year graduate student at the University of Hawaii, Mr. Kit-Kwan Lee, translated pertinent sections. Algae, surprisingly, constitute only a modest portion of the slim book. The prescriptions for the preparations of the medicines are often precise, while the medical applications tend to be broad and perhaps somewhat expansive. For example, a concoction made of 250 g of *Porphyra dentata* and 250 g of *Cassia tora* seeds (a terrestrial plant, family Leguminosae) is said to cure hypertension; or a soup made of *P. suborbiculata* will cure irritability, tuberculosis, goiter, toothache, and hypertension, as well as invigorating the kidney, expelling phlegm, and promoting diuresis.

INVERTEBRATES

The largest section of the publication from China (South China Sea Institute of Oceanology 1978) is devoted to the medicinal uses of invertebrates. At first sight this seems surprising since fishes and shelled molluscs are the most widely known marine biota. Closer examination, though, shows that intensive use is indeed made of the shells of edible molluscs. A few examples illustrate this point. Shell of abalone (*Haliotis* spp.) heated, ground, and often mixed with a variety of terrestrial herbs has been used to cure, *inter alia,* eye inflammation, hepatitis, and hypertension. *Murex triremis* shell, calcined, mixed with borneol and ground, will cure otitis. A soup from boiled mussel shell (*Mytilus viridis*) will cure dizziness, impotence, and premature ejaculation. It will also stop bleeding and act as a tranquilizer. In some cases the entire animal, flesh and shell, is pounded and boiled. The bivalve *Meretrix meretrix,* after such preparation, will cure tuberculosis, diabetes, kidney and eye disease.

Invertebrates other than molluscs are not overlooked. The shell of the crab *Calappa philargius,* after slow baking, grinding, and mixing with millet wine is an oral contraceptive. (There is no indication whether it works for male, female or both sexes.) Nematocysts of the Portuguese man-of-war (*Physalia physalis utriculus*) can be extracted to isolate a cardio-active drug. The boiled gorgonian *Melitodes squamata,* when taken by mouth, will cure tuberculosis or stop frightened children from being scared. Otitis may be cured by grinding the spines of the sea urchin *Heterocentrotus mammillatus* with vinegar and pouring the suspension in the affected ear. A soup made by boiling the dried sea star *Craspidaster hesperus* in water will cure goiter.

Organized and published information on the medicinal use of marine invertebrates in countries other than China is scarce. Titcomb, who carried out extensive researches on Hawaiian cultural practices, focussed on food rather than medicine. In her paper (Titcomb 1978) on the use of marine invertebrates, she mentions a few medicinal aspects in passing. A siliceous sponge, *Leiodermatium* sp., may have been used "to cure the white fur on the tongue." Black coral (*Antipathes grandis*), mixed with many other ingredients, was used for "lung trouble and for kindred diseases." The dried tentacles of the terebellid worm *Lanice conchilega,* mixed with water, are said to be a cancer remedy. Attempts to isolate the active constituent(s) were made (Tabrah et al. 1970; Norton et al. 1973). The crab *Lybia edmondsoni* was considered poisonous, and sometimes used as a heart stimulant.

Perhaps the best known inedible marine invertebrate from Hawaii is the anthozoan *Palythoa toxica* (Fig. 1; Moore and Scheuer 1971; Moore et al. 1982). I suspect that it is this animal, the so called *limu make-o-Hana* (the deadly seaweed of Hana) that prompted Dr. William Fenical to invite a speaker from Hawaii to talk about ethno-natural historical leads. This is, indeed, an example *par excellence* of such a lead that has had, and continues to have, an impact on biomedicine and related sciences. Determination of its complex and unique molecular structure in Hawaii (Moore and Bartolini 1981) and Japan (Uemura et al. 1981), elucidation of its three-dimensional architecture (Cha et al. 1982; Fujioka et al. 1982; Klein et al. 1982; Ko et al. 1982) are intellectual milestones in the modern history of organic chemistry. Its laboratory synthesis and its utilization as an effective anti-cancer agent, first adumbrated more than ten years ago (Quinn et al. 1974) continue to be major research challenges. Of interest may be a letter to the editor of the newspaper *Ka Lahui Hawaii,* published on 23 August 1877.

> Muolea, Hana, Aug. 11, 1877
>
> Editor, Greetings.
>
> Please permit me to tell something of the poisonous sea weed of Muolea, at Hana, East Maui.
>
> In olden times it did not grow as it does now and the natives who lived near the sea pools did not know that it was poisonous. When some children went to the sea pools to catch ohua fish to eat, those who ate a quantity became dizzy and fainted by the pools. They revived when medicine was administered. After that, a man from Honaunau in Kona, Hawaii discovered it. When the pigs ate sweet potatoes he went to fetch the sea weed and rubbed it over the potatoes. After the pigs came back to eat them, every single one died. When the dogs went to lick the vomited matter from the dead pigs, they too died. That is how they found out that it was poisonous, for it also grows in Honaunau, Hawaii.
>
> If you should pick it up with your fingers, they will rot and break off. The only thing to do is to poke it up with a stick and lay it down on a ti or taro leaf. As soon as you touch it it shrinks and wilts like a sensitive plant. It is not long like other algae and is like the suckers on an octopus. On certain kapu nights of the year, a red glow is seen where it is found
>
> In A.D. 1841 perhaps, the sea pool was filled with stones but now more is growing and out toward the open flats. The fish that swim around it are not harmed, but if you eat the fish of the sea pools, you will die.
>
> This is the fastest working poison like the deadliest haole poison and perhaps more potent. For this reason any person who has the right to, is absolutely prohibited from going there.
>
> With thanks to the printers and my love to the Editor.
>
> Abraham Kauhi

This account is noteworthy in that it mentions a Honaunau site on the island of Hawaii for occurrence of *P. toxica,* and for a report of fish that ingest the coral and are dangerous to eat. During the modern investigation of *Palythoa* spp., a filefish (*Alutera scripta*), was observed by Hashimoto et al. (1969) to contain palytoxin. More recently Yasumoto (personal communication from Professor T. Yasumoto, Tohoku University) examined toxic fish from Micronesia, which contained palytoxin.

FISHES

In her comprehensive treatment of native use of fish in Hawaii, Titcomb (1972) makes no mention of any direct medicinal use of fish. She briefly reports the use of fish by medical practitioners (*kahunas*) as a kind of coda at the end of a prescribed course of therapy. She alludes to ciguatera intoxications and describes in some detail the elusive mullet poisoning that is said to cause nightmares. This has not been substantiated by controlled experiments at the Hawaii Institute of Marine Biology.

By contrast with the dearth of information on the medicinal use of fish in Hawaii, the Chinese monograph (South China Sea Institute of Oceanology 1978) devotes some 40 pages to this subject. Among the more fascinating prescriptions are the use of the backbone of *Rhincodon typus,* the whale shark, said to be the largest of all fishes, to cure headaches when it is stewed with chicken or rock candy and eaten. Cancer of the esophagus and the stomach can be cured by eating the dried and ground

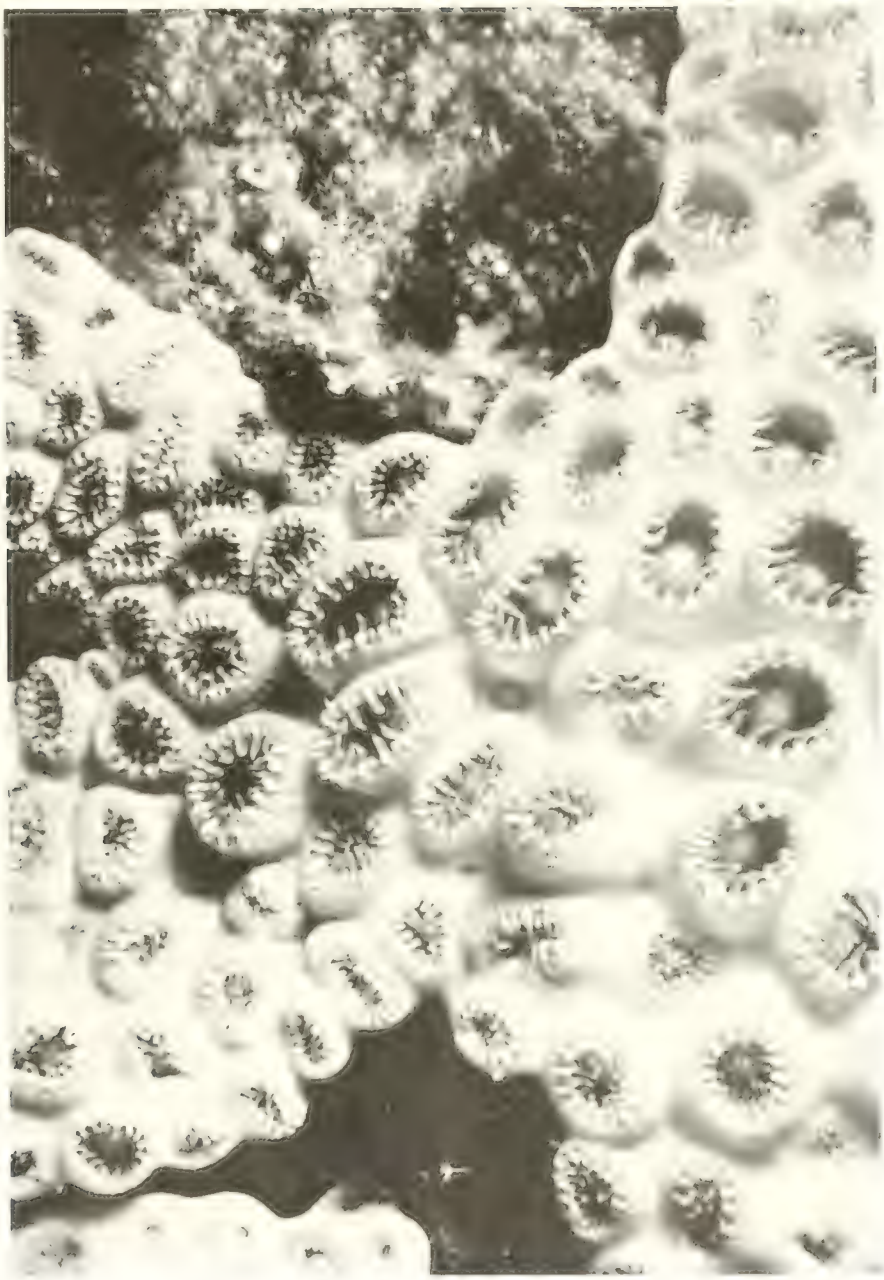

FIGURE 1 Polyps of *Palythoa tuberculosa*. Photograph by Dr. Mark Yunker

spine of a stingray *Dasyatis akajei,* after it is mixed with sesame oil or rice vinegar. A moray eel, *Gymnothorax reticularis,* when ashed, mixed with millet wine, and eaten, will cure hemorrhoids. To cure impotence, sterility, and insomnia, one removes the viscera of *Syngnathus acus* (a pipefish), fries it with honey, grinds it, mixes it with millet wine, and eats it. Flesh from a fish with the intriguing name *Inimicus japonicus,* belonging to the venomous scorpionfishes, when freshly cooked with corn whiskey (do not add water!) will cure abdominal pain. Another fish, well-known for its toxic potential, *Fugu vermicularis,* is used to reduce swelling of the lymph glands. Fresh ovary and liver are pounded and the resulting mash is applied externally. It is mentioned that injection of fugu poison (tetrodotoxin, presumably) causes sedation and has analgesic properties. Pure tetrodotoxin, which has been commercially available for the past 20 years, selectively blocks sodium channels of nerve membranes and has been used as a probe for the study of neurophysiological mechanisms.

CONCLUSION

This attempt to uncover ethno-natural historical leads to marine biomedicine has confirmed my initial suspicion that their number is small by comparison with terrestrial biomedicine. Surprisingly, though, they do exist and only a few have been followed up with modern techniques. It remains to be seen what

the results might be when a greater effort is made to use ethno-natural history as a guide toward biomedical discoveries in the marine ecosystem.

ACKNOWLEDGMENT

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Uniqueness of the Marine Chemical Environment: Categories of Marine Natural Products from Invertebrates

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INTRODUCTION

Marine organisms are a source of great fascination for a multitude of people, ranging from scientists who study the sea in an attempt to understand the forces that control our world, to the youth who collects sea shells as great treasures. Among the scientists who have studied the sea and its inhabitants are the marine natural product chemists whose interests lie in understanding secondary metabolic processes of marine organisms. These organisms have provided a rich harvest of secondary metabolites as attested to by the volume of published reports in the literature. Between 1977 and 1985 some 1,700 compounds were described from marine sources. These have been comprehensively reviewed by Faulkner (1984*a*, *b*, 1986). There have also been several selective reviews covering topics such as marine alkaloids (Fenical 1986) and diterpenes (Fenical 1978), as well as the proposed biological significance of these metabolites (Scheuer 1978). This review will focus on the diversity and novelty of secondary metabolites isolated from marine invertebrate animals and will include a discussion of the unique characteristics of the marine environment that may contribute to the breadth of secondary metabolism exhibited by these animals. This review is intended to be a brief overview of selected examples principally from the phyla Coelenterata, Porifera, Chordata, Bryozoa, and one example from the Echinodermata. The intent is to show the variety of marine natural products but will, to a degree, reflect the interest of this research group in nitrogenous metabolites. The Mollusca will be excluded partly because they tend to be consumers rather than producers of secondary metabolites, and also because that topic is dealt with in this volume by D. J. Faulkner.

The world's oceans cover greater than 70% of the earth's surface, and, taking into account volume, the oceans represent better than 95% of the biosphere (Barth and Broshears 1982). All but two of the 28 principal phyla in the animal kingdom are represented in aquatic environments; eight phyla including the Coelenterata, Porifera, Bryozoa, and Echinodermata are exclusively aquatic, largely saline in habitat. Greater than 95% of all animal species are invertebrates, and a conservative estimate is that there are over 200,000 species represented in the world's oceans (George and George 1979). It is generally accepted that the invertebrates—and the animal kingdom in general—had their origins in the primordial oceans (Barth and Broshears 1982). Consequently, the animals that never left this environment have had a longer time period to adapt to their present environment. Also, because of the ability of sea water to moderate changes in salinity, pH, and temperature it can be argued that marine organisms live in a more uniform and stable environment than their terrestrial counterparts (Barnes 1980). Although it is difficult to provide unequivocal proof, conceptually it can be argued that marine invertebrate organisms, having propagated over eons in a more stable environment, have had the luxury of diverting greater amounts of resources to the development of secondary metabolic pathways, as part of their chemical survival strategy. Marine invertebrates have spawned an impressive array of unique biological and chemical adaptations in response to their environment. One such adaptation, to the lack of organic nutrition that is wide spread in the marine environment, is the development of symbiotic relationships between invertebrates and photoautotrophic algae. The algae provide the animal host with photosynthetically fixed organic carbon, and, in some cases, fixed nitrogen. Examples include reef building corals, which harbor eukaryotic zooxanthellae, and didemnid tunicates that harbor a prokaryotic alga.

There is a variety of circumstantial evidence to support the claim that the production of secondary metabolites represents a range of adaptive survival mechanisms, including the accumulation of toxic metabolites of dietary origin in dorsal glands by nudibranch molluscs (Faulkner and Ghiselin 1983), production of antifouling metabolites by bryozoans (Al-Ogily and Knight-Jones 1977), secretion of trail–following and –breaking allomones by marine molluscs (Cook and Cook 1975), and the secretion of toxic metabolites by sponges and soft corals (Coll et al. 1985) to kill neighboring organisms. It has been suggested by others that natural products may be a form of metabolic waste or dead-end products of an ancestral biochemical pathway, now obsolete (Haslam 1986). Nevertheless, one must consider whether the production and accumulation of natural products by marine invertebrates (often a large percentage of the biomass) is not without significant cost to the producer, and whether this function would have been conserved throughout the evolutionary process had it not conferred some adaptive advantage to the producer. If so, the frequency of biological activity (up to 82% of sponge compounds are antimicrobial) (Rinehart et al. 1981*c*) may not be a mere coincidence but instead reflect a meticulous natural selection that is just now becoming obvious to us.

In contrast to terrestrial natural products studies, which have focused largely on secondary metabolism of prokaryotic microbes, fungi, and higher plants, the majority of natural products (58%) isolated from marine organisms since 1977 have come from invertebrate animals (e.g., sponges, coelenterates, and tunicates). Given the inherent biochemical differences between animals and plants, combined with the environmental and evolutionary factors discussed above, it should not be surprising that many marine invertebrates elaborate biosynthetic products previously unreported from terrestrial sources.

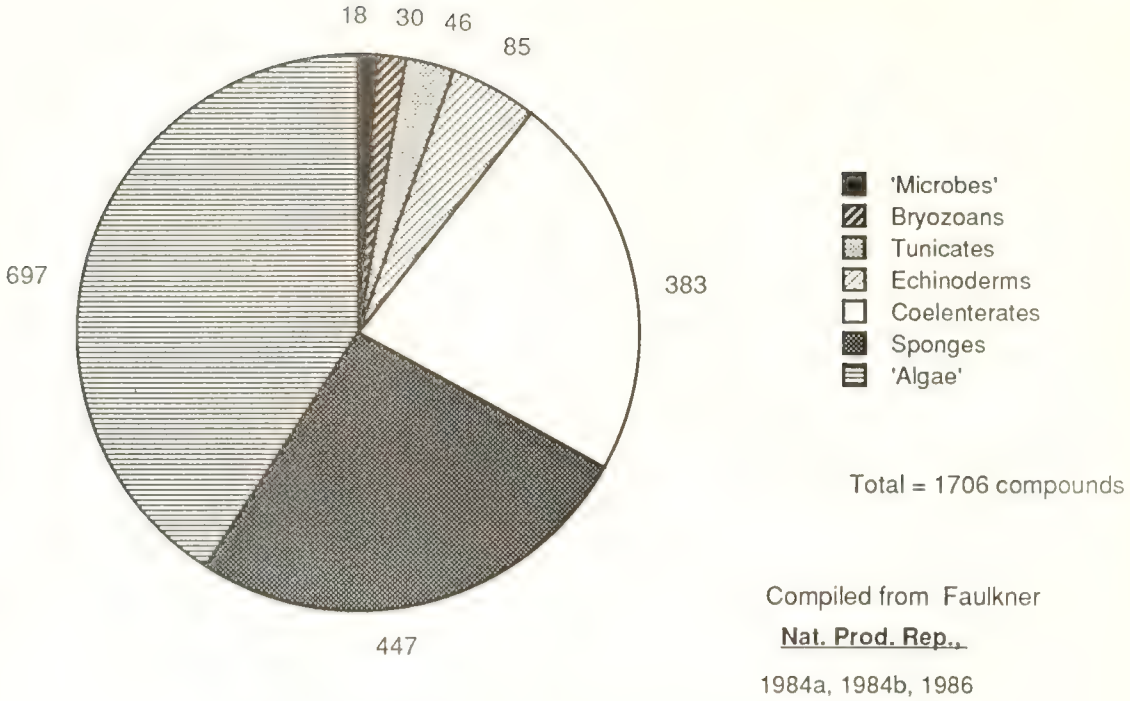


FIGURE 1. Phyletic distribution of marine natural products.

DISTRIBUTION OF MARINE NATURAL PRODUCTS

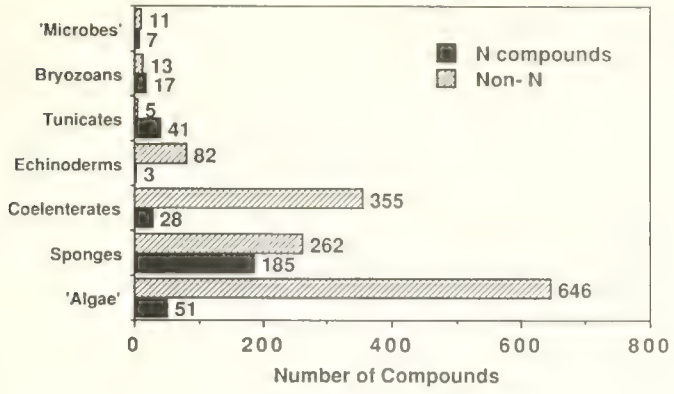
A phyletic distribution of marine natural products reported between 1977 and 1985, as compiled from data in recent reviews (Faulkner 1984*a, b,* 1986), shows some interesting trends. Although algae as a single group are responsible for the largest percentage of natural products, invertebrate animals as a group account for 58% of the approximately 1,700 metabolites reported during this eight year period (Fig. 1). Figures 2 and 3 provide a graphical breakdown of the total number of metabolites and the percentage of nitrogenous metabolites isolated from marine organisms in the same period. The three largest divisions (algae, coelenterates, and sponges), together with the tunicates, are further analyzed and the distribution of the classes of compounds in these groups is shown in Figure 4. These numerical analyses are biased in that they cover only the last eight years, and reflect the foci of the individual research groups currently involved in the field. Nevertheless, the figures profile the current interest in the field and demonstrate some pertinent differences between the secondary metabolism of marine plants and animals, as well as among individual phyla.

Figure 3 expands the algae data presented in Figure 2 to include the various algal classifications. There is a clear dominance of non-nitrogenous metabolites isolated from both the red and brown algae. In contrast to this, the distribution of nitrogenous and non-nitrogenous metabolites is much more equitable in the phytoplankton and blue-green algae. Characteristic differences in secondary metabolism are not limited to the algae, and are also seen between the animal phyla as well (Fig. 4).

The coelenterates have been a prolific group of marine invertebrates yielding ~400 secondary metabolites (Fig. 2). They are also the most consistent from the point of view that 85% of coelenterate metabolites are terpenoid, principally from the octocorals, while the 7% of metabolites that contain nitrogen are produced mostly by the zoanthids (Fig. 4). This is not to say that coelenterates are uninventive chemists. In fact, the coelenterates are responsible for the production of 14 new skeletal classes of terpenes (Fig. 5), as well as members of known terpene skeletons with unique substitution patterns and functionalities. They are also the source of a group of modified prostaglandins and the most renowned marine natural product, palytoxin.

Sponges, the oldest and most primitive metazoans, are prolific producers of both terpenoid and alkaloid metabolites. Approximately 37% of the 447 sponge metabolites reported between 1977 and 1985 are terpenoid, with an additional 6% being of mixed biosynthesis, in part involving the terpene pathway (Fig. 4). Nitrogenous metabolites account for 41% of sponge metabolites, including the rare isonitriles and unusual purine and pyrimidine derivatives.

The tunicates are members of the phylum Chordata. In contrast to the coelenterates, the tunicates specialize in amino acid metabolism, with greater than 89% of their metabolites containing nitrogen (Fig. 2). The total number of metabolites isolated from tunicates is relatively small (~50), but this includes some very important examples from the viewpoint of biological activity. A number of tunicate species belonging to the family Didemnidae harbor a prokaryotic unicellular symbiont that shares many characteristics with blue-green algae (Lewin and Withers 1975). The occurrence of this symbiont, and the fact that blue-green algae are themselves a source of many unique natural products—approximately 59% of them nitrogenous (Fig. 3)—has prompted speculation as to the ultimate source of tunicate chemistry. This speculation, however, must be tempered



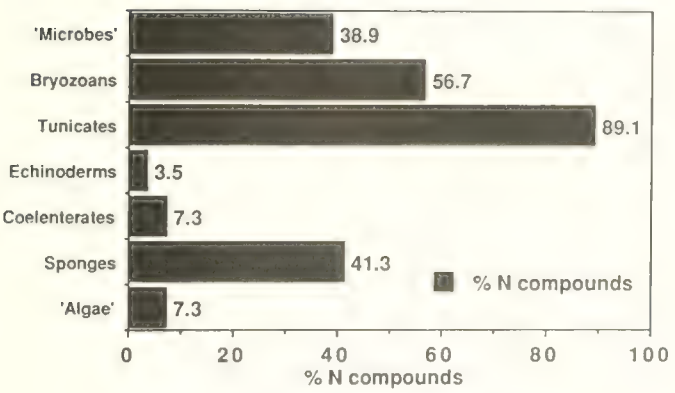
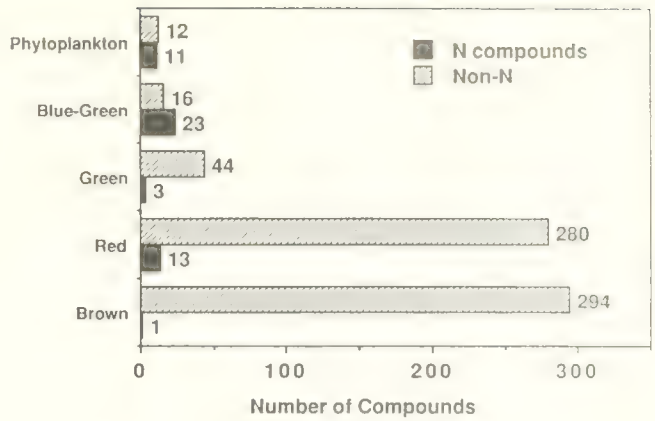


FIGURE 2. Distribution of nitrogenous compounds.



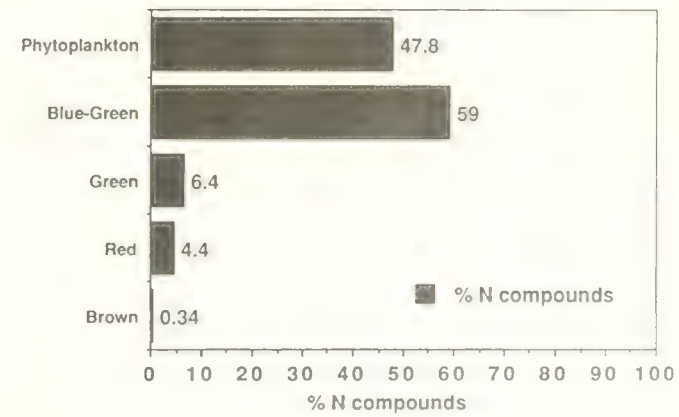


FIGURE 3. Distribution of nitrogenous compounds in algae

by the observation that not all didemnids that have yielded interesting chemistry harbor symbionts, and many amino acid metabolites such as the eudistomins have been isolated from non-didemnid tunicate species.

The phylum Bryozoa has been little studied by marine natural products chemists but has nonetheless yielded some very rewarding chemistry. These generally small colonial organisms that are commonly found in tropical waters yielded only 30 metabolites—more than half of which are nitrogenous—during the eight-year period between 1977 and 1985 (Fig. 2). Numbered among this group are the bryostatins and several new classes of alkaloids.

COELENTERATES

The principal terpenoids elaborated by coelenterates are sesquiterpenes and diterpenes. An example of both the diversity and antipodal nature of coelenterate sesquiterpenes are those isolated from the *Sinularia* soft corals. One species alone, *Sinularia mayi,* elaborates more than half a dozen different sesquiterpene skeletons, e.g., **1–6**. All of these are enantiomeric to their most prevalent terrestrial counterparts (Beechan et al. 1978).

Clavularia species have also yielded a variety of unusual sesquiterpenes, some of which are related to *Sinularia* compounds. These include the acetoxysinularins **7** and **8** from *C. inflata* (see Braekman et al. 1981). However, the most interesting *Clavularia* terpenes are the C_{12} compounds clavukerin A (**9**) and inflatene (**10**) isolated from *C. koellikeri* and *C. inflata* var. *luzoniana,* respectively. Clavukerin A was the first naturally occurring 2,8-dimethylhexahydroazulene derivative isolated. Inflatene is a potent ichthyotoxin, active against *Pomacentrus coeruleus* (a Pacific damselfish) at 10 μg/ml. Its structure was proposed based on COSY and ^{13}C-^{1}H difference nOe NMR measurements and confirmed by the synthesis of two derivatives of inflatene (Izac et al. 1984).

Two additional sesquiterpene skeletons that were previously unknown are the capnellane and *ent*-valerenane skeletons isolated from *Capnella* sp. and an unidentified Xeniidae species, respectively. The capnellanes, which have three five-membered rings fused in series (e.g., **11**), have antifeedant activity against *Lebistes reticulatus* (common guppy) (Kaisin et al. 1985). They also proved to be powerful inhibitors of algal growth at minute concentrations (Tursch 1976). There was no biological data reported for the *ent*-valerenanes (e.g., **12**).

The diterpene skeleton most frequently elaborated by coelenterates is the cembranoid system, which contains a 14-membered carbocycle. Unlike their terrestrial counterparts, the coelenterate cembranes are often highly functionalized and oxidized. More than 100 cembranes have been isolated from coelenterates, many with biological activity. Crassin acetate (**13**), from

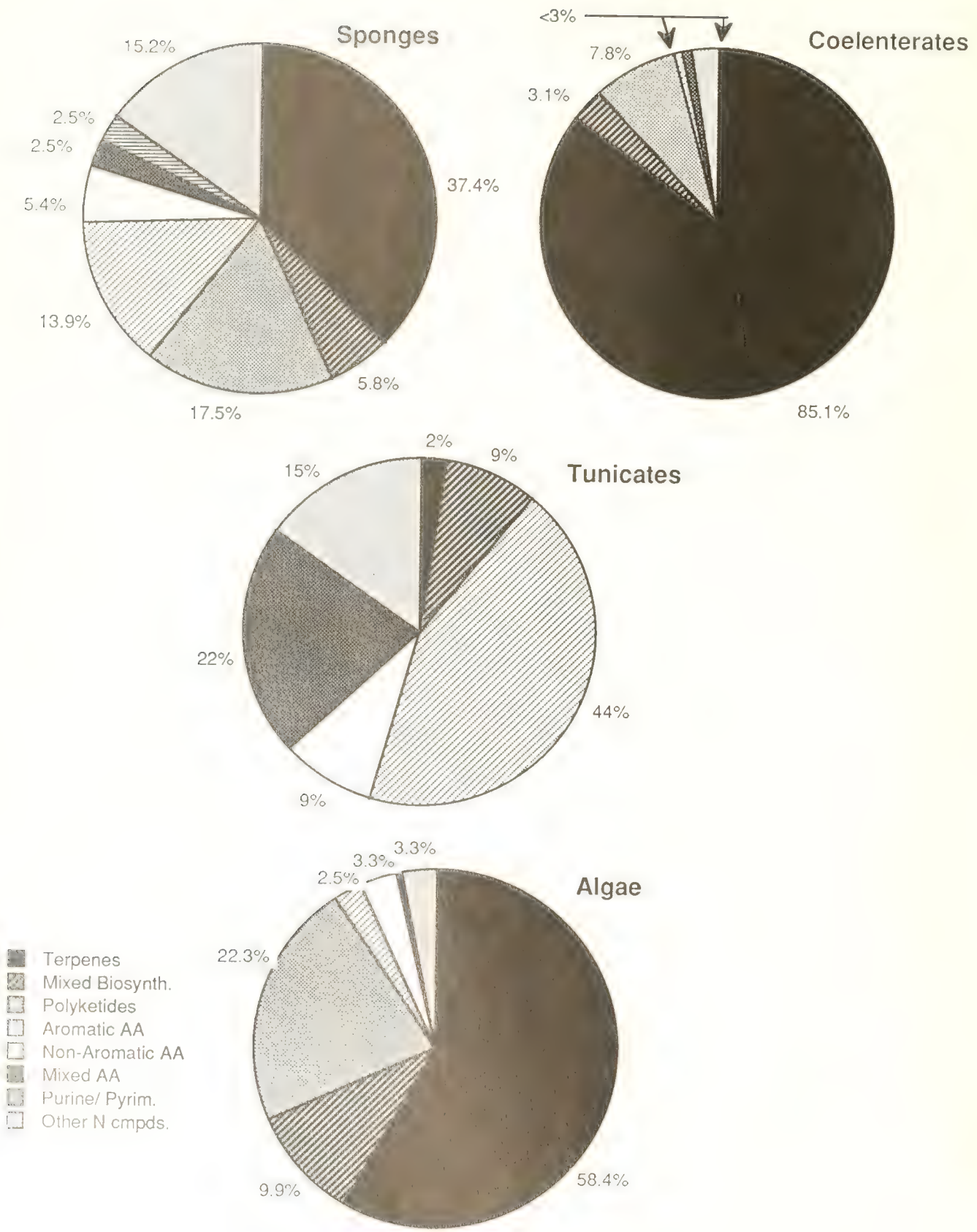


FIGURE 4. Distribution of marine natural products by class

Pseudoplexaura sp., one of the earliest cembranes to be reported, has an α-methylene-δ-lactone fused to the carbocyclic system, and was found to inhibit P388 leukemia in vivo (T/C 130 at 50 mg/kg) (Weinheimer and Matson 1975). A closely allied compound sinularin (=flexibilide) (**14**), isolated from *Sinularia flexibilis,* showed in vitro activity against KB and P388 cells with an ED_{50} of 0.3 and 16 μg/ml, respectively (Weinheimer et al. 1977). Sinularin was also shown to kill the hard corals *Acropora formosa* and *Porites andrewsii* at concentrations of 2–5 ppm, and at sub-lethal concentrations showed significant effects on photosynthesis and respiration of *A. formosa* (see Coll et al. 1985).

An example of a more highly functionalized cembrane is lophotoxin (**15**), which was isolated from several sea whips of the genus *Lophogorgia* (see Fenical et al. 1981). Lophotoxin is a potent neurotoxin (LD_{50} 8.0 μg/ml, IP mice), which causes ataxia, paralysis, and severe respiratory depression, followed by death. Lophotoxin contains rare α,β-epoxy-γ-lactone and furanoaldehyde functionalities, which are believed to be largely responsible for its toxic activity. Structure–activity relationship studies of lophotoxin and its congeners suggest that lophotoxin binds covalently to the nicotinic receptors by way of a Michael addition or by Schiff base formation (Culver et al. 1985). The epoxylactone and furanoaldehyde functionalities are found separately in only a few other natural products, and this is the first report of their presence in a marine natural product. Lophotoxin co-occurs with the related compound pukalide (**16**), which lacks the epoxylactone functionality (Missakian et al. 1975) and shows almost no neurotoxic activity, supporting the importance of that functionality for neurotoxic activity.

Although the cembranes are the most commonly isolated diterpenes, many non-cembranoid diterpenes have also been reported. Xenicin (**17**), isolated from *Xenia elongata,* represents the first example of this skeleton (Vanderah et al. 1977). Xeniaphyllenol (**18**), which contains a [7.2.0] undecane (caryophyllene) skeleton, was also isolated from *X. elongata* (see Groweiss and Kashman 1983). The calyculones A–C (**19–21**), isolated from *Eunica calyculata,* are examples of cubitane diterpenes. The structures of the calyculones were determined exclusively by spectroscopic and chemical methods (Look et al. 1984). The more highly oxidized cubitane pseudopterolide (**22**) was isolated from the sea whip *Pseudopterogorgia acerosa* (see Bandurraga et al. 1982).

The *Briareum* soft corals elaborate chlorinated diterpenes, which possess the novel briarein skeleton. Briarein A (**23**), from *B. asbestinium,* was the first example of this group reported (Burks et al. 1977). Briarein Y (**24**) exhibits insecticidal activity against *Melanopus bivattatus* (grasshopper) (LD_{50} < 3 mg) and is toxic to *Salmonella* strains without signs of mutagenicity, even at concentrations of 7 μg/ml (Cardellina et al. 1984).

The pseudopterosins **25–28** are diterpene glycosides isolated from the sea whip *Pseudopterogorgia elisabethae,* and belong to the rare amphilectane skeletal class (Look et al. 1986*a*). It should be noted that nonsteroidal glycosides are rare among marine natural products. The pseudopterosins possess antiinflammatory activity equivalent to or greater than indomethacin and appear to work by an as yet undefined mechanism of action. They do not appear to inhibit either cyclooxygenases or lipoxygenases, and have limited effects on phospholipase A_2s (Look et al. 1986*b*).

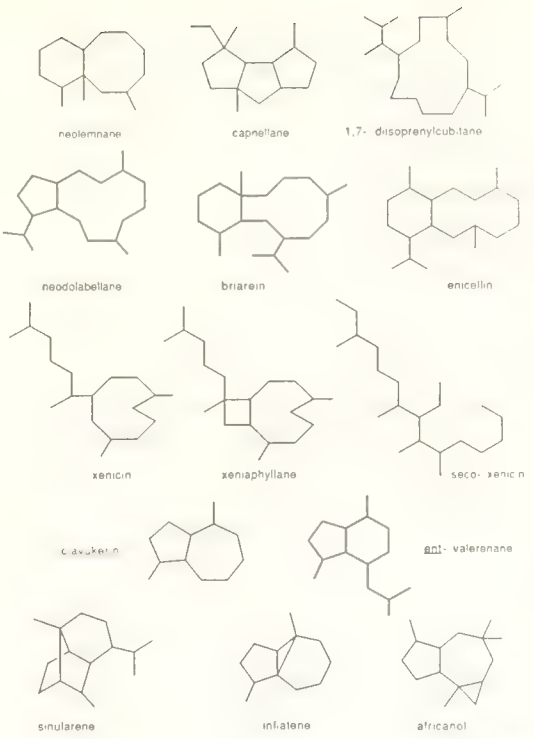


FIGURE 5. Parent skeletons from novel coelenterate terpenes.

Early reviews of coelenterate chemistry speculated about the possible role(s) of terpenes, suggesting they could be defensive allomones; namely, antifeedants or ichthyotoxins. Extensive studies on aqueous extracts from soft corals have shown that only 50% of the common, exposed soft corals were toxic. However, half of the nontoxic extracts possessed significant antifeedant activity. In addition, an inverse relationship between physical defense and toxicity was observed (Coll et al. 1985).

Besides their ichthyotoxic/antifeedant properties, soft coral terpenes have been isolated in the water column surrounding individual colonies and subsequently were shown to provide a competitive advantage against neighboring organisms. An example cited earlier is sinularin (**14**), which has been shown to kill competing hard corals (Coll et al. 1985).

In 1969, Weinheimer and Spraggins reported the first isolation of a prostaglandin, (15R)-PGA_2, from the gorgonian *Plexaura homomalla.* This represented the first example of a prostaglandin from a nonmammalian source. Subsequently, several additional prostaglandins were reported from *P. homomalla.* The first prostanoid isolated from a soft coral was a PGF_2 derivative **29** from *Lobophytum depressum* (see Carmely et al. 1980). Shortly thereafter, the clavulones (e.g., **30**) were isolated from *Clavularia viridis.* The clavulones represented the first prostanoids with oxygen functionalities at C4, C12, and olefins at C7 and C14. The clavulones showed significant antiinflammatory effects at 30 μg/ml in the fertile egg test, which uses the chorio-allantoic membrane of the chick embryo as the site of induced inflammation (Kikuchi 1982*b,* 1983*b*).

Later, the claviridenones (e.g., **31**) were isolated from *C. viridis* (see Kobayashi et al. 1982, 1983). Finally, a group of cytotoxic prostanoids—the punaglandins—characterized by a C12 oxygen

and C10 chlorine, were isolated from the octocoral *Telesto riisei*. Punaglandin 3 (**32**) showed significant activity against the L1210 cell line with an IC_{50} of 0.02 μg/ml (Baker et al. 1985).

Another group of compounds with antiinflammatory activity are the butenolides **33–36** isolated from *Euplexaura flava*. The butenolides are the first long chain fatty acid derivatives from a marine source to possess a rare three-carbon branch at the α-position. They showed significant antiinflammatory effects at 100 μg/ml in the fertile egg test (Kikuchi et al. 1982*a*, 1983*a*). A well-defined group of nitrogenous metabolites from coelenterates are the zooanthins **37** and pseudozooanthins **38** isolated from *Parazoanthus* sp. and *Epizoanthus* sp. (Cariello et al. 1974; Schwartz et al. 1978). Paragracine (**39**) (Komada et al. 1982, 1984), a bioactive pseudozooanthin, showed papaverine-like activity (papaverine is a non-specific smooth muscle relaxant). Paragracine also selectively blocks Na^+ channels of squid axon membranes in a frequency-dependent manner. This blocking action occurs from the cytosolic side of the membrane and paragracine enters the channel as Na^+ leaves the axon (Seyama et al. 1980).

Studies on zoanthids have yielded several interesting nitrogenous metabolites. Zooanthamine (**40**) is an alkaloid isolated from a colonial species of *Zoanthus* collected intertidally along the coast of India. This species was chosen for study because zoanthids are well-documented producers of skin and eye irritants. Zooanthamine was the first metabolite isolated and, although it is not a skin irritant, it nevertheless represents a new alkaloid class (Rao et al. 1984).

The structure elucidation of palytoxin (**41**) is one of the triumphs of natural products research. Palytoxin, a metabolite of the zoanthid *Palythoa toxica* and other *Palythoa* species (Moore and Scheuer 1971), is the most potent non-proteinous toxin ever isolated. Its reported LD_{50} of 0.15 μg/kg makes it 50 times more toxic than tetrodotoxin and saxitoxin. Palytoxin has a molecular formula of $C_{129}H_{223}N_3O_{54}$, has no repeating unit, and contains many functional groups along its backbone (Uemura et al. 1980; Moore and Bartolini 1981; Moore 1982).

Sponges

The interest in sponge metabolites has been credited to Bergmann and Feeney (1950) with the discovery of the nucleosides spongouridine (**42**) and spongothymidine (**43**) from *Tethya crypta*. These compounds served as models for the synthesis of Ara-C (**44**), a nucleoside analogue with antiviral and antitumor properties. A more recent representative of these pharmacologically active nucleosides is 1-methylisoguanosine (**45**), isolated from the Australian sponge *Tedania digitata* (see Quinn et al. 1980). This compound exhibited potent skeletal muscle relaxant activity, antiinflammatory, and antiallergy properties. It also produced cardiovascular effects similar to those of adenosine and may indeed function as an adenosine analogue (Baird-Lambert et al. 1980).

A novel antitumor alkaloid, manzamine A hydrochloride (**46**), was reported from the Okinawan sponge *Haliclona* sp. (Sakai et al. 1986). An x-ray analysis of the natural product revealed a unique array of 5-, 6-, 8-, and 13-membered rings joined together in an unprecedented skeletal arrangement. Manzamine A hydrochloride exhibited an IC_{50} of 0.07 μg/ml against P388 mouse leukemia cells. The authors reported that there is no obvious biogenetic pathway for the formation of this unusual ring system.

The Caribbean *Tedania ignis*, often called the fire sponge, yielded the potent cytotoxic macrocycle tedanolide (**47**) (Schmitz et al. 1984), which is one of a variety of polyketide metabolites isolated from sponges. Tedanolide, representing a mixed acetate-propionate biogenesis, is highly cytotoxic and exhibits an ED_{50} of 2.5×10^{-4} μg/ml in KB and 1.6×10^{-5} μg/ml in PS cell lines.

A 22-membered polyketide macrolide has been isolated from the Red Sea sponge *Theonella swinhoei* (see Carmely and Kashman 1985). The structure of swinholide (**48**), which possesses *in vitro* antifungal activity, was determined by 2D NMR experiments conducted on a tetraformate derivative. Several nOe experiments established the diene configuration while NMR ^{13}C-^1H and ^1H-^1H correlation spectroscopy confirmed the partial structures (an α,γ-diunsaturated ester, an allyl ether, a 1,3-diol, a THP ring, and a CH(Me)CH(OH)CHMe moiety), and established connections between them. The macrolide structure **48** was unambiguously confirmed by the long-range coupling seen between the lactone carbonyl and the lactonic methinoxy group, which also allowed definitive assignments of the methoxyl and ether carbons. This work clearly illustrates the power and utility of 2D NMR spectroscopy in the structure elucidation of complicated natural products.

Another Red Sea sponge, *Latrunculia magnifica*, was the first marine organism to yield 14- and 16-membered macrolides, to which an uncommon 2-thiazolidinone ring is attached (Kashman et al. 1980). *Latrunculia* is a conspicuous red sponge that has never been observed to be eaten by fish. The sponge produces a red juice when squeezed that causes an immediate avoidance response in fish. The compounds responsible for this activity were identified as latrunculins A (**49**) and B (**50**). Latrunculin B differed from A in that it contained a 14- versus 16-membered macrocycle. Latrunculin A had an LD_{50} of 0.4 mg/l toward the mosquito fish, *Gambusia affinis* (see Neeman et al. 1975). The toxin caused erratic behavior in the fish followed by hemorrhaging, loss of balance, and death. These symptoms suggested that the toxin may have some effect on the nervous system of the fish, and indeed, *in vitro* experiments revealed that latrunculin A is a cholinesterase inhibitor. The latrunculin's effect on cultured mouse neuroblastoma and fibroblast cells is to cause changes in cell morphology that are reversible upon removal of the toxins (Spector et al. 1983). The toxin's effects are similar to those of the cytochalasins, mold metabolites that disrupt the cell's microfilamentous structures. However, the site of action of the latrunculins is different.

An extremely unusual family of antitumor polyether metabolites has been reported from *Halichondria* and *Pandaros* sponges. Halichondrin B (**51**), one of eight halichondrins isolated from *H. okadai*, exhibited potent *in vivo* antitumor activity against B16 melanoma (T/C 244 at 5.0 μg/kg), P388 leukemia (T/C 236 at 5.0 μg/kg), and L1210 leukemia (T/C 207 at 50 μg/kg) (Hirata and Uemura 1986). The authors suggest that the halichondrins may act as detergents, inserting into lipid bilayers and disrupting membrane integrity. They propose that the unprecedented trioxatricylco [3.3.2.0] decane serves as a non-polar head group and that the terminus should include two or three hydroxyl groups for the molecule to demonstrate significant antitumor activity. Okadaic acid (**52**), isolated from *H. okadai*

and independently from *H. melanodocia* collected in the Florida Keys (Tachibana et al. 1981), is a desulfur derivative of the episulfide acanthifolicin (**53**) found in the isopropanol extract of *Pandaros acanthifolium* from the Virgin Islands (Schmitz et al. 1981). These metabolites were the first polyether carboxylic acids of marine origin and, in addition, the episulfide in acanthifolicin was an unprecedented feature among the published polyether antibiotics. Both **52** and **53** contain C_{38} backbones, the largest carbon backbone of any reported polyether antibiotic. Acanthifolicin exhibits ED_{50}s of 2.8×10^{-4} (P388), 2.1×10^{-3} (KB), and 3.9×10^{-3} μg/ml (L1210) (Schmitz et al. 1981); okadaic acid is slightly less toxic having ED_{50}s of 1.7×10^{-3} (P388) and 1.7×10^{-2} μg/ml (L1210) (Tachibana et al. 1981).

Calyculin A (**54**), a unique antitumor metabolite encompassing a rich array of functionalities, has been isolated from the marine sponge *Discodermia calyx*, collected from the Gulf of Sagami (Kato et al. 1986). Phosphorus-31 NMR spectroscopy verified the presence of a phosphate ester, and 2D NMR spectroscopy was used to construct partial structures for calyculin A. However, the structure of **54** was only unequivocally assigned by x-ray analysis. Calyculin A inhibited the development of starfish embryos at 0.01 μg/ml and exhibited a cytotoxicity IC_{50} value of 1.75×10^{-3} μg/ml against L1210 cells. The unprecedented C_{28} backbone of 54 is connected to two γ-amino acids and incorporates oxazole, nitrile, phosphate, spiro ketal, and amide functionalities not normally found in the same molecule.

Of all the various metabolites produced by sponges, the most abundant and widely distributed are of terpenoid origin. The variety of terpene skeletons is exemplified in the next several metabolites discussed. Manoalide (**55**) is a sesquiterpenoid antibiotic obtained from the Palauan sponge *Luffariella variabilis* (see de Silva and Scheuer 1980). The CH_2Cl_2 extract of *Luffariella* demonstrated potent *in vitro* activity against *Streptomyces pyogenes* and *Staphylococcus aureus*. Manoalide represents a new class of pharmacological agents, exhibiting both analgesic and antiinflammatory properties. Manoalide, which acts as an inhibitor of phospholipase A_2, showed analgesic effects at 50 mg/kg in the phenylquinone test and antiinflammatory activity better than indomethacin in a phorbol myristate acetate assay (Jacobs et al. 1985).

Gracilin B (**56**) is an unusual bis-norditerpene from the Mediterranean *Spongionella gracilis* (see Mayol et al. 1985). The structure determination of 56 was based on spectral analysis and relied heavily on the use of 2D ^{13}C-^{1}H NMR chemical shift correlations to observe two- and three-bond couplings. The structure of gracillin B was elucidated by LAH reduction and acetylation to give the pentaacetate derivative **57**. The stereochemistry was assigned by nOe difference spectroscopy and examination of molecular models (Mayol et al. 1985, 1986). The highly oxygenated gracilin B represented the first report of a bis-norditerpene from a marine sponge.

A new triterpene, bearing an novel pentacyclic skeleton, has been isolated from the Red Sea sponge *Siphonochalina siphonella* (Carmely and Kashman 1986). The structure of neviotine-A (**58**) was deduced by chemical transformations and the analysis of 2D INADEQUATE NMR data. The authors performed 2D INADEQUATE experiments that distinguished all of the C-C connections except C12-C13 and C20-C21, which were designated by a 2D RELAY experiment. The RELAY experiment also confirmed the position of the benzoxepine ring and completed the assignment of the carbon backbone. Many sesquiterpenoid, and more recently diterpenoid, metabolites have been isolated from sponges belonging to the orders Axinellida, Halichondrida, and Haplosclerida. One of the first examples from this area of natural products research was the isolation and structure determination of 9-isocyanopupukeanane (**59**) from the Hawaiian sponge *Hymeniacidon* sp. (Burreson et al. 1975) (this sponge was later identified as a *Ciocalypta* sp. [Gulavita 1986]). The structures of both **59** and the 2-isomer **60** (Hagadone et al. 1979) were determined by x-ray analysis and were responsible for rekindling an interest in the biosynthetic origin of the isonitrile moiety. The most highly functionalized terpenoid isonitrile to be characterized to date is kalihinol-A (**61**) (Chang et al. 1984). This tricyclic diterpene also contains hydroxyl, tetrahydropyranyl, and chlorine moieties. Kalihinol-A exhibited in vitro activity against *Bacillus subtilis, Staphylococcus aureus*, and *Candida albicans*. Kalihinol-A contains an unrearranged diterpenoid skeleton but is unique in the number of different functional groups present on the ring. Four other kalihinols—B, C, E, and F—have now been isolated from *Acanthella*, kalihinol-E (**62**) being the C14 epimer of **61**. Kalihinol-F (**63**), an unprecedented triisocyano diterpenoid with a tetrahydrofuranyl ring, also inhibited the growth of *B. subtilis, S. aureus*, and *C. albicans* (Patra 1984).

Since the isolation of the first naturally occurring isocyano metabolite, xanthocillin (**64**), from *Penicillium* in 1957 (Hagedorn 1957), scientists have been intrigued by the origin of the isonitrile functionality. Labelling experiments furnished evidence that tyrosine was responsible for the xanthocillin skeleton, but neither formate nor methionine were precursors of the isonitrile group (Achenbach 1967). With the isolation of the related formamide, isothiocyano, and isocyanopupukeananes, speculation grew that the formamides might be the antecedents of the isocyano group. However, it has now been demonstrated that the formamide and isothiocyano groups are biosynthesized from the isonitrile (Hagadone et al. 1984). Recent studies with the sponge *Amphimedon*, which elaborates diterpene isonitriles, demonstrated incorporation of sodium [^{14}C]cyanide into the isonitrile functionality (Garson 1986). There are still many unanswered questions about the biosynthesis of the isonitrile group, and this will certainly be an active research area in the future.

Although peptides represent a small group of sponge metabolites, several unusual and bioactive examples have been reported. *Discodermia kiiensis* from the Izu Archipelago of Japan contained the first antimicrobial peptides to be isolated from a sponge (Matsunaga et al. 1984, 1985*a, b*). Discodermin A (**65**) inhibited the growth of *B. subtilis, S. aureus, Escherichia coli*, and *Pseudomonas aeruginosa* at 1 μg/disk. The tetradecapeptide structure of **65** was determined by Edman degradation of the desformyl compound (Ala-Phe-Pro-t-Leu-t-Leu-Trp) and analysis of the BNPS-skatol reaction product (H-Arg-Cys(O_3H)-Thr-MeGln-Leu-Asn-Thr-Sar). EI mass spectrometry fragmentation patterns were then used to assign the structures of discodermins B–D (**66–68**), which differ only in residues four and five from the N-terminus (Matsunaga 1985*b*). In addition to antibacterial activity, the discodermins inhibited the development of starfish embryos, the minimum concentration for **65** and **67** being 5 μg/ml. Discodermin A contains two t-Leu residues, which had previously been found only in Actinomycete metabolites.

A novel depsipeptide containing a 12-carbon polypropionate

unit has been independently isolated from *Jaspis* species collected in Fiji and Palau (Crews et al. 1986; Zabriskie et al. 1986). Jaspamide (**69**) exhibited potent insecticidal activity (LC_{50} = 4 ppm) against the tobacco budworm *Heliothis virescens*, and also inhibited the growth of *Candida albicans* at 1 μg/disk. Alanine and β-tyrosine residues were assigned on the basis of 2D ^1H and ^{13}C NMR data, as was the structure of the novel amino acid 2-bromoabrine. Hydrolysis of **69** and subsequent derivatization using dansyl chloride produced an alanine residue having the (S) configuration as determined by chiral HPLC. Structure confirmation and determination of the relative stereochemistry were unambiguously established by x-ray analysis of the corresponding O-acetate. Jaspamide exemplifies a new class of cyclic depsipeptides, and is unusual in that it contains a polypropionate subunit and two rare amino acids, both of which possess (R) stereochemistry.

An interesting characteristic of some sponge orders has been their conformity to chemotaxonomic patterns. A prime example of consistency lies within the order Verongida, in which every species examined produces secondary metabolites derived from the amino acid bromotyrosine. The Australian *Ianthella basta* manufactures such a series of seven novel metabolites, the bastadins, containing four bromotyrosine units (Kazlauskas et al. 1980, 1981). Bastadin-2 (**70**) and bastadin-6 (**71**) are members of this series, which show potent *in vitro* antimicrobial activity against Gram-positive bacteria. Another series of compounds, aplysinopsin (**72**) (Kaslauskas et al. 1977) and the 6-bromo derivatives **73** (Tymiak et al. 1985) and **74** (Djura et al. 1980), derived from the amino acid tryptophan, have been isolated only from dictyoceratid sponges. The Australian *Thorecta* (=*Aplysinopsis*) sp. yielded aplysinopsin as yellow needles. Two 6-bromo derivatives of aplysinopsin, **73** and **74**, were isolated from the Caribbean *Smenospongia aurea*. Their structures were determined by spectral comparisons with **72**. Aplysinopsin exhibited mild cytotoxic activity against three cell lines, displaying ED_{50}s of 0.87 μg/ml, 3.8 μg/ml, and 3.7 μg/ml against KB, P388, and L1210 cell cultures, respectively (Hollenbeak and Schmitz 1977).

Tunicates

The simplest non-nitrogenous metabolites from a tunicate are the prenylated quinone derivatives isolated from *Aplidium*. Prenylhydroquinone **75**, 6-hydroxy-2,2-dimethylchromene **76**, and prenylquinone **77** were isolated from *Aplidium californicum* (see Howard and Clarkson 1979). Compound **75** showed *in vivo* activity against P388 leukemia (T/C 138 at 3.12 mg/kg), while both **75** and **77** significantly inhibited the mutagenic effects of benzo(a)pyrene, aflatoxin B_1, and ultraviolet light on *Salmonella typhimurium*. A second group of non-nitrogenous metabolites is represented by the pentenones (**78–81**) isolated from *Didemnum voeltzkowi* from Fiji (Sesin and Ireland, unpublished), and **82** from an unidentified didemnid from the Caribbean (Lindquist and Fenical, unpublished). The epimeric nature of **79** and **80** was demonstrated by the fact that allylic oxidation of **79** produced γ-lactone **83** indicating *cis* stereochemistry of the substituents at C4 and C5. Treatment of **80** under identical conditions gave aldehyde **84**, which failed to form a lactol, thus indicating a trans relationship at C4 and C5. Compounds **78–81** exhibited *in vitro* activity against the murine leukemia L1210 with IC_{50} values in the 5–0.5 μg/ml range.

The largest group of metabolites reported from tunicates is the amino acid derivatives, having two subgroups, the alkaloids and peptides. Dendroine (**85**), a tryptophan alkaloid that contains a novel 3-N,N-dimethyl-1,2,4-thiadiazole moiety, was isolated from *Dendrodoa grossularia*, and its structure was determined by x-ray diffraction (Heitz et al. 1980). Dendroine exhibits activity *in vitro* against the L1210 leukemia, and is the first example of a S(II)-N bond in a natural product.

Eudistoma olivaceum has been the source of a large family of β-carboline alkaloids called the eudistomins (**86–103**) (Kobayashi et al. 1984; Rinehart et al. 1984; Kinzer and Cardellina 1987). To date there are 18 members of this family, which can be grouped into five subclasses based on the substitution at Cl. Biosynthetically, these represent condensation of tryptophan with proline (A, G, H, I, M, P, Q), a modified cysteine that forms an unusual oxathiazepine ring (C, E, K, L), and a phenyl pyruvate (R, S, T). Several of the eudistomins exhibit weak antimicrobial and antiviral activity. However, eudistomins-C, -E, -K, and -L display significant activity against *Herpes simplex* virus-1 (C, 50 ng/disk; E, 50 ng/disk; K, 250 ng/disk; L, 100 ng/disk) (Kobayashi et al. 1984; Rinehart et al. 1984).

The first peptides to be isolated from a tunicate were the Lissoclinum peptides from *Lissoclinum patella* (see Ireland and Scheuer 1980; Ireland et al. 1982; Hamamoto et al. 1983; Wasylyk et al. 1983; Sesin et al. 1986). There are now 12 members of the family, all of which are cyclic and contain at least one thiazole and usually an oxazoline amino acid. There are no terrestrial counterparts to the Lissoclinum peptides. The Lissoclinum peptides can be placed into three subgroups based on structure: Ulithiacyclamide (**104**); the patellamide group that includes patellamides A (**105**), B (**106**), and C (**107**), and ascidiacyclamide (**108**); and the lissoclinamide group, which encompasses lissoclinamides 1 (**109**), 2 (**110**), and 3 (**111**), and ulicyclamide (**112**). All but one member of the family, ascidiacyclamide, were isolated from *L. patella* collected at Palau. Several new structure determination methods were developed as part of this study. These include a method for establishing the absolute configuration of thiazole amino acid based on the reaction of thiazoles with singlet oxygen to form a cycloadduct which, upon hydrolysis, gives an α-amino acid. A large majority of the thiazoles possess the (R) absolute configuration (Biskupiak and Ireland 1983). A new method for sequencing small peptides, based on the observation of homoallylic coupling between α-protons of α-amino acids using a COSY-45 experiment, was also reported (Sesin et al. 1986). The structures of the patellamides (A–C) were corrected (the original assignment placed the thiazoles attached to C-2 of the oxazolines) based on the observation of 5-bond couplings between the α-oxazoline and α-thiazole protons in the COSY-45 spectra. This coupling, not detectable in the conventional one-dimensional spectrum, was determined to be less than 0.2 Hz. Structures **105–107** were also confirmed by synthesis (Hamada et al. 1985; Schmidt and Griesser 1986; Schmidt and Weller 1986). Ulithiacyclamide is the most potent of the Lissoclinum peptides exhibiting *in vitro* anticancer activity against L1210 (0.1 μg/ml), HeLa (0.1 μg/ml), and CEM (0.01 μg/ml) cell lines, and *in vivo* activity against the P1534J murine leukemia (T/C 188 at 1 mg/kg, repetitive doses).

The didemnins A–C (**113–115**) are a new class of cyclic depsipeptides isolated from the Caribbean tunicate *Tridemnum* sp. (Rinehart et al. 1981*a, b*). The didemnins contain a new structural component for depsipeptides, hydroxyisovalerylpropionate (HIP), and the new allo stereoisomer of statine. The structures of the didemnins were based, in great part, on interpretation of field desorption mass spectrometric data. The stereochemistry of the HIP unit was recently revised to 2S, 4R (Ewing et al. 1986). In addition to their structural novelty, the didemnins also exhibited impressive *in vitro* and *in vivo* antiviral activity. Didemnins A and B inhibited *Herpes simplex* viruses 1 and 2 at 1.0 μM and 0.05 μM concentrations, Rift Valley fever virus at 1.37 and 0.04 μg/ml, Venezuelan equine encephalomyelitis virus at 0.43 and 0.08 μg/ml, and yellow fever virus at 0.4 and 0.08 μg/ml. Mice infected with Rift Valley fever showed 90% survival when treated with didemnin B at 0.25 mg/kg. There were, however, some drug related deaths at this dose (Canonico et al. 1982). Didemnin B has also demonstrated *in vivo* anticancer activity against P388 murine leukemia (T/C 199 at 1 mg/kg) (Rinehart 1981*b*). Didemnin B was subsequently evaluated *in vitro* against human tumors in a stem cell assay (Jiang et al. 1983). Tumor cells from eight of 17 patients showed sensitivity to didemnin B with a median IC_{50} of 4.2×10^{-3} μg/ml. Didemnin B is currently in phase 2 of human clinical trials as an anticancer agent. Added to the impressive list of activities associated with didemnin B is its effect as an immunosuppressive agent. In a Simonsen parental-to-F_1 graft-versus-host assay, didemnin B showed 71% inhibition of splenomegaly with repetitive doses at 0.3 mg/kg (Montgomery 1985).

Ascidia nigra, a black Floridian tunicate, sequesters vanadium as the pentavalent vanadate, concentrates it 10^6-fold, and stores it as the reduced V(III) or V(IV) states at physiological pH. The instability of V(III) at pH greater than 2.5 is apparently overcome by complexing the metal with a strongly reducing species. The tunichromes, a series of bright yellow pigments from tunicate blood, are reportedly responsible for this activity. The structure of one of these pigments, tunichrome B-1 (**116**), was recently established as a modified dopa peptide (Bruening et al. 1985, 1986). The tunichromes, which are sensitive to air and water, were purified by centrifugal counter-current chromatography carried out under dry O_2-free argon using completely degassed solvents. Approximately 6,000 tunicates yielded 0.5 mg of pure tunichrome B-1.

Bryozoans

The predominant chemistry associated with bryozoans involves tryptophan metabolism. The simplest examples are two highly brominated gramine derivatives **117** and **118** isolated from *Zoobotryon verticullatum*. Compound **118** represents the first example of an N-oxide from a marine organism, and both compounds inhibited cell division of fertilized sea urchin eggs at approximately 16 μg/ml (Sato and Fenical 1983).

The cold water bryozoan *Flustra folicacea* has yielded a variety of indole derivatives including the flustrabromines **119** and **120** (Wolff et al. 1981) and a family of physostigmine alkaloids called the flustramines (e.g., **121**) (Carle and Christophersen 1979). The flustrabromines were determined to be rotational isomers based on the fact that the NMR spectra began to coalesce between 35 and 50°C. Similar results were reported for N-acetyl-N-methyl tryptamine (Wolff 1981). A second cold water species, *Chartella papyracea*, which belongs to the same family as *Flustra*, yielded the unusual indole alkaloid chartellamine A (**122**) (Chevolot et al. 1985). Chartellamine A is a pentahalogenated, pentacyclic metabolite that represents a new class of tryptophan alkaloids. Chevolot proposes a biosynthesis involving condensation of a prenylated tryptamine with a modified histamine. Unfortunately, there is no biological testing data reported for chartellamine.

The family Bugulidae has also proven to be a rich source of diverse and biologically potent metabolites. *Sessibugula translucens* is the source of a family of bipyrroles called the tambjamines (**123–126**), which were originally isolated from three different species of nudibranchs (Carte and Faulkner 1983). The details of the metabolic history of these compounds are not completely understood. However, it has been proposed that the bryozoan obtains prodigiosin (**127**) from the bacterium *Benecea* and subsequently converts it into the tambjamines. The tambjamines exhibit antimicrobial activity against a variety of microbes including *E. coli, S. aureus, V. anguillarum*, and *C. albicans* at the 1–5 μg/ml level. The tambjamines appear to be utilized by the nudibranch *Tambje* as part of a defensive secretion. The compounds are secreted in a yellow mucus when the animal is attacked. A blue pigment with antimicrobial activity was recently isolated from *Bugula dentata* and identified as tetra-pyrrole (**128**) (Matsunaga et al. 1986).

The most important and unusual class of bryozoan metabolites are the bryostatins, isolated originally from *Bugula neritina*, and subsequently from *Amathia convoluta*. The bryostatins are a family of macrocyclic lactones all with the same unprecedented 26 membered "bryopyran" skeleton (Pettit and Herald 1982, 1983*a, b*; Pettit and Kamano 1985). The eight members in the family differ principally in the ester groups linked to the macrocyclic lactone ring. Bryostatins-1, -2, and -3 (**129–131**), for example, showed significant *in vivo* anticancer activity in the PS protocol (1:T/C 159–196 at 10–70 μg/kg, 2:T/C 160 at 30 μg/kg, 3:T/C 163 at 30 μg/kg).

A final example of bryozoan chemistry is phidolopin (**132**), an unusual purine base isolated from *Phidolopora pacifica*. Phidolopin, which possesses a rare nitrophenyl ring, showed *in vitro* antifungal activity against *Pythium ultimum, Rhizoctonia solani*, and *Helminthosporium satium* when tested at a concentration of 70 μg/disk (Ayer et al. 1984).

Echinoderms

Imbricatine (**133**), from the sea star *Dermasterias imbricata*, is one of only a handful of non-saponin metabolites isolated from echinoderms. The structure determination was accomplished by spectroscopic methods including INAPT experiments to determine two and three bond couplings, as well as degradation studies and syntheses of model compounds (Pathirana and Anderson 1986). This is the first example of a benzyltetrahydroisquinoline alkaloid from a non-plant source. In addition, the C3 carboxy, the C6/C8 hydroxylation substitution pattern, and the thioester linkage to histidine represent new functionalities in this alkaloid family. This compound induces "swimming behavior" at low concentrations (1–2 drops

solution with a concentration 1 mg/ml) in the sea anemone *Stomphia coccinea*. It also displays significant *in vitro* antineoplastic activity against the L1210 and P388 cell lines with ED_{50} <1 μg/ml and T/C 139 at 0.5 mg/kg, respectively.

CONCLUSIONS

There is ample evidence that marine organisms are an important source of new classes of "bioactive" metabolites, and judging from the activity in this field (1,700 compounds reported in eight years) will continue to be so for at least the next decade. A number of these compounds have found application as probes for new mechanisms of action and several are under development as pharmaceutical agents. Perhaps the major drawback to pharmaceutical development in this field is that marine organisms have not proven amenable to either laboratory culture or harvest. For these reasons it appears that many pharmaceutical companies have shied away from major investments in this field. This lack of industrial support is also responsible, in part, for the narrow focus of investigations in this field—principally towards cytotoxic, antimicrobial and more recently, antiviral activity. Some investigations into antiinflammatory, cardiovascular, and immunoregulator activities of marine products have been underwritten partially by industrial support.

The greatest controversy (or unanswered question) within the discipline has been the origin of metabolites isolated from invertebrates that are associated with unicellular microbes. This question was first raised with regard to the metabolism of octocorals that harbor zooxanthellae, as well as to sponges, which play host to significant bacterial populations. In the case of octocorals, it now appears clear that the symbionts are not directly involved in production of secondary metabolites, although they undoubtedly provide primary metabolites to the host. In regard to sponges, it is much less clear. A variety of circumstantial evidence suggests, in selected cases, bacteria or other prokaryotic organisms are responsible for production of compounds. This includes compounds that have previously been reported from terrestrial microbes or are minor modifications of known microbial products. It has also been argued that compounds that are isolated in very small quantities (0.001% or less of dry weight) are products from a contaminating microbe. Unfortunately, there is no unequivocal evidence to support these claims. However, there is a great deal of activity in this area as well as in related areas involving tunicates, and it appears to only be a matter of time before hard evidence is provided.

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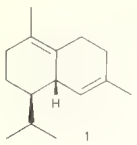
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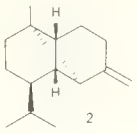
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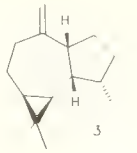
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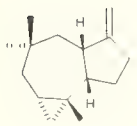
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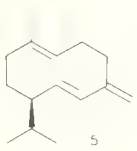
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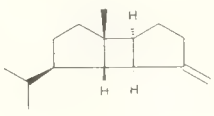
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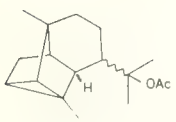
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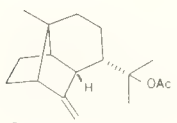
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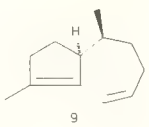
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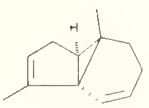
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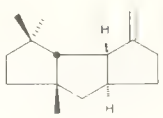
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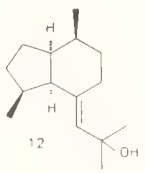
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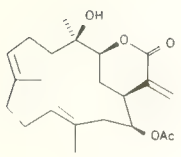
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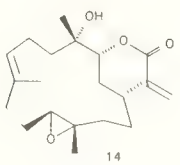
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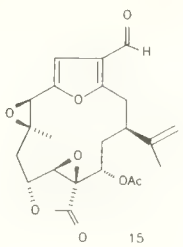
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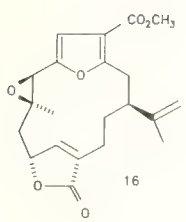
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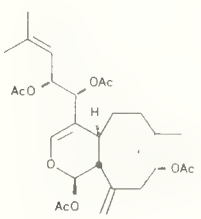
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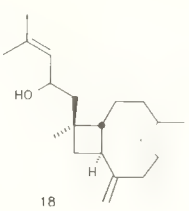
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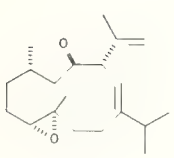
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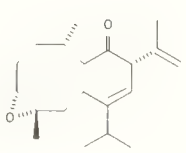
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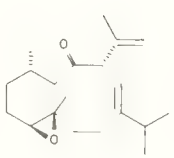
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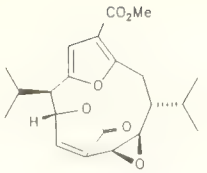
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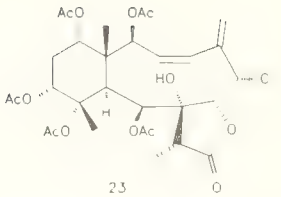
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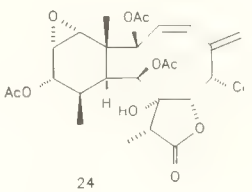
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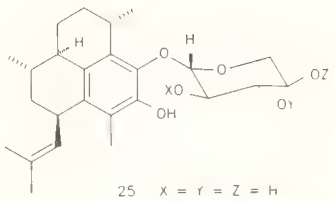
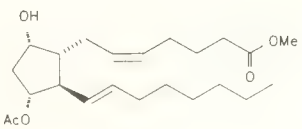

25 X = Y = Z = H
26 X = Ac, Y = Z = H
27 X = Z = H, Y = Ac
28 X = Y = H, Z = Ac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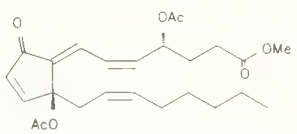
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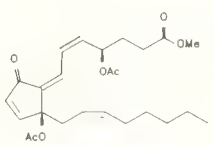
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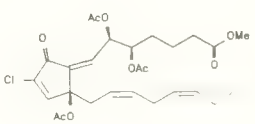
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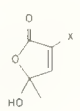
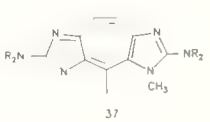

33 X = $C_{18}H_{33}$
34 X = $C_{20}H_{37}$ (6Z, 8Z diene)
35 X = $C_{22}H_{39}$ (6Z, 8Z, 10Z triene)
36 X = $C_{22}H_{37}$ (6Z, 8Z, 10Z, 12Z tetraene)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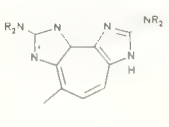
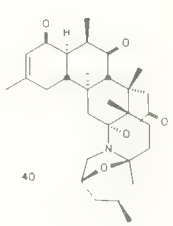

38
39 R = Me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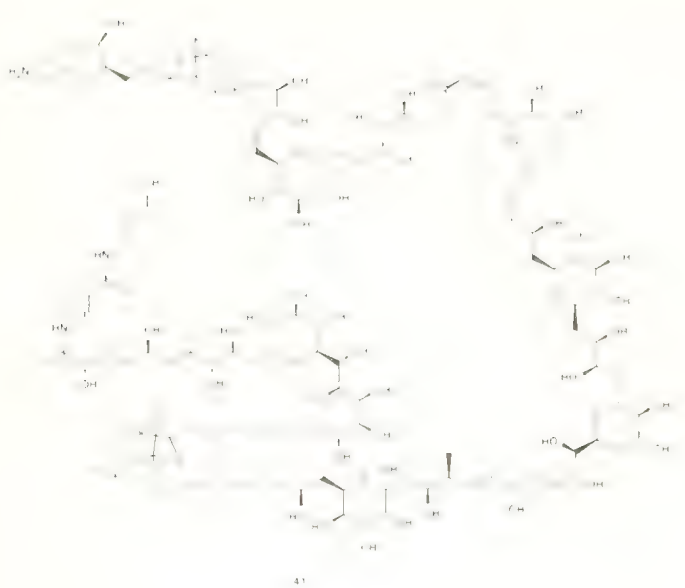

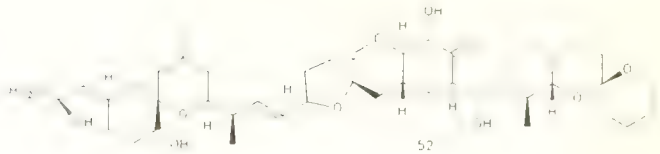

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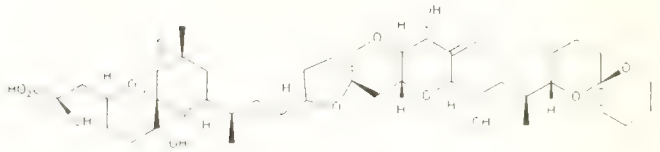

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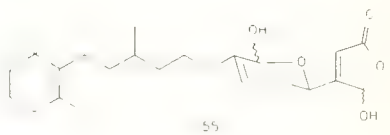

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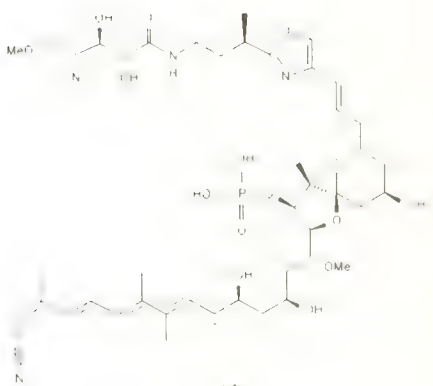

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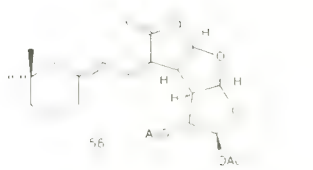

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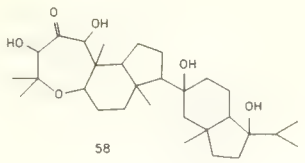

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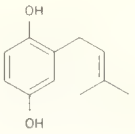

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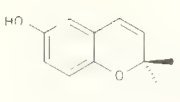

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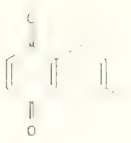

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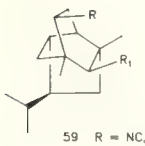
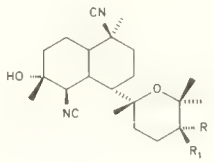
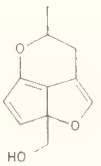


59 R = NC, R_1 = H
60 R = H, R_1 = NC



61 R = Cl, R_1 = H
62 R = H, R_1 = Cl



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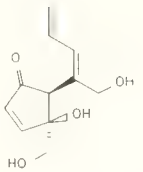

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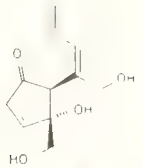

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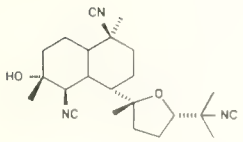
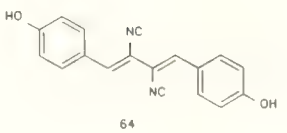


63



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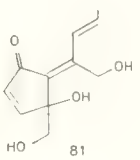

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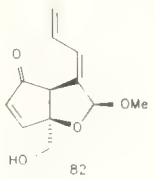

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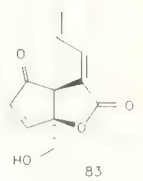
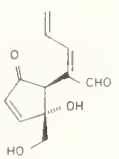


83

HCO-D-Ala-L-Phe-L-Pro-X-D-Trp-L-Arg-D-Cyc(O_3H)-L-Thr-L-MeGln-D-Leu-L-Asn-L-Thr-Sar

65 A: X= D-t-Leu-L-t-Leu
66 B: X= D-Val-L-t-Leu
67 C: X= D-t-Leu-L-Val
68 D: X= D-Val-L-Val



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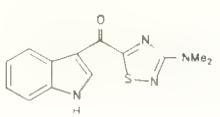

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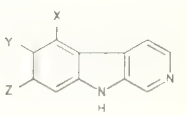
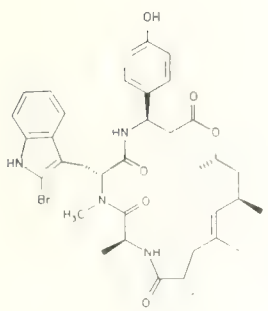


86 D X = Br, Y = OH, Z = H
87 J X = H, Y = OH, Z = Br
88 N X = Z = H, Y = Br
89 O X = Y = H, Z = Br



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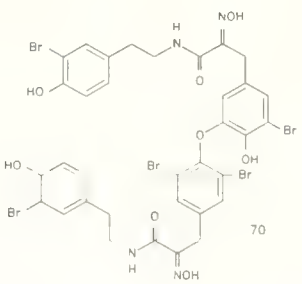

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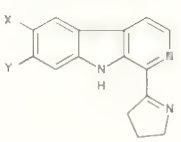
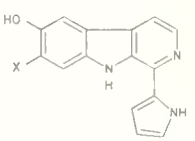
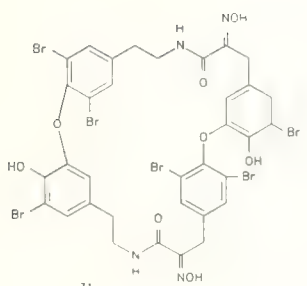


90 G X = H, Y = Br
91 H X = Br, Y = H
92 I X = Y = H
93 P X = OH, Y = Br
94 Q X = OH, Y = H



95 A X = Br
96 M X = H



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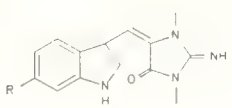
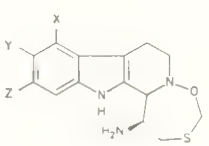
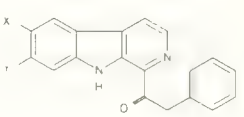


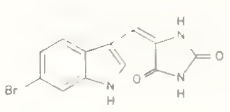
72 R = H
73 R = Br



97 C X = H, Y = OH, Z = Br
98 E X = Br, Y = OH, Z = H
99 K X = Y = H, Z = Br
100 L X = Z = H, Y = Br



101 R X = H, Y = Br
102 S X = Br, Y = H
103 T X = Y = H



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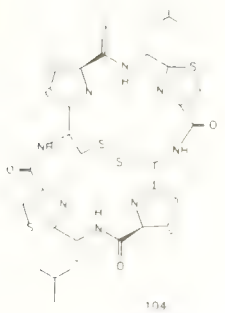

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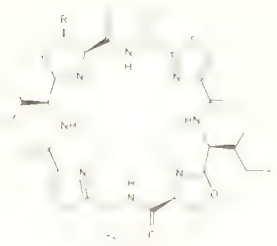
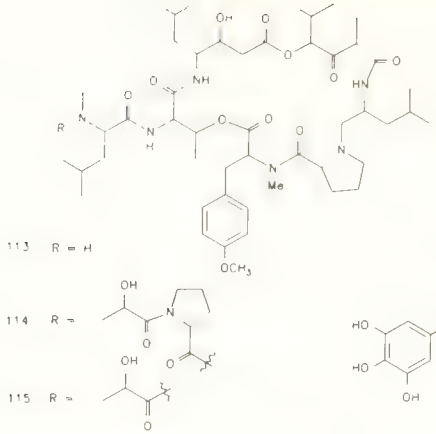


135 R = CH_3

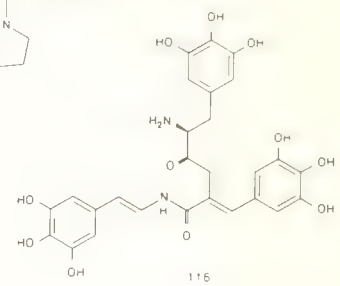
108 R = H



113 R = H

114 R =

115 R =



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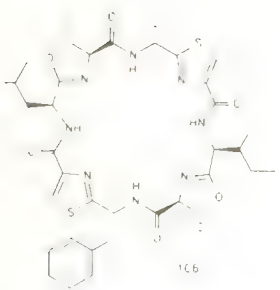

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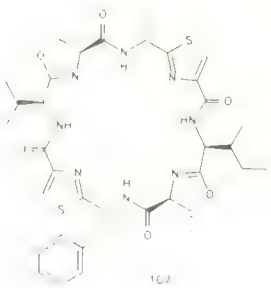

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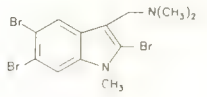

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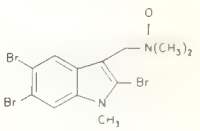

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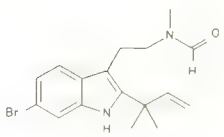

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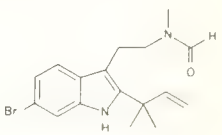

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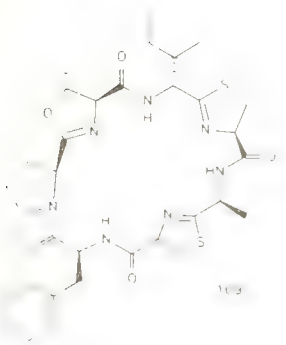

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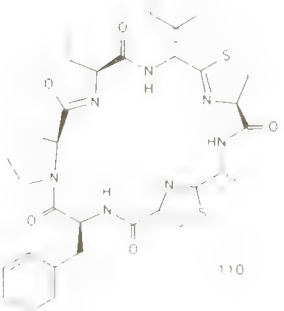

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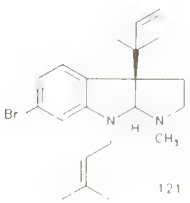

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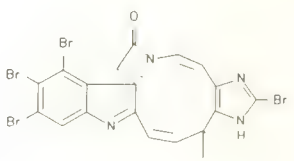

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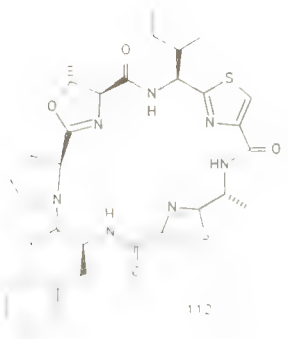


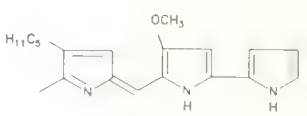
112

123 X = H, Y = H, Z = H

124 X = Br, Y = H, Z = H

125 X = H, Y = H, Z = iBu

126 X = H, Y = Br, Z = iBu



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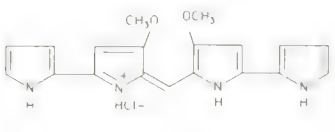

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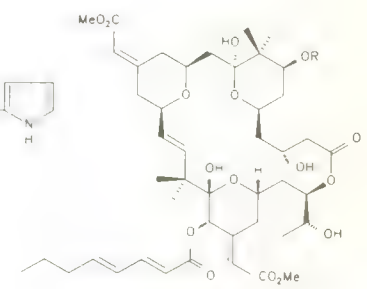
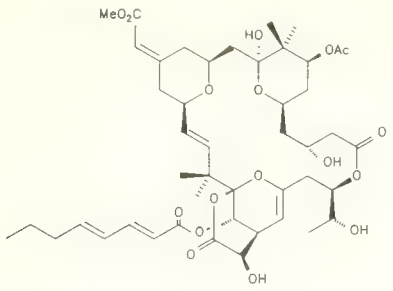
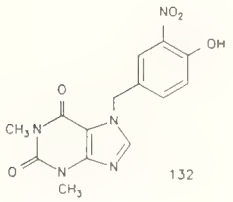


129 R = Ac

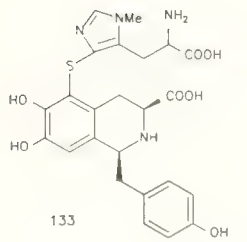
130 R = H


MeO2C
HO
OAc
O
O
O
OH
O
O
O
O
O
OH
O
OH

131


NO2
OH
O
CH3 N
N
O
N
N
CH3

132


NMe
NH2
N
COOH
S
HO
COOH
HO
NH
OH

133

Characterization of Factors that are Intimately Involved in the Life of Marine Organisms

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INTRODUCTION

Despite the current world-wide interest in drugs from the sea, this area is still largely untouched. In contrast to our interest in terrestrial natural products, including Chinese and other folk medicine, which have been known for thousands of years, our knowledge of medicinally important natural products from the sea is extremely limited. However, the recent renewed interest in this area, following a hiatus in the 1970s, is indeed promising and exciting.

The key step in discovering compounds of biomedical interest, independent of whether they are of terrestrial or of marine origin, lies in isolation monitored by bioassay. There are two general approaches: one is to screen for general activities such as antineoplastic or antibiotic, while the other is to focus on "biological factors," which are intimately related to the maintenance of life of that species. Structural elucidation of the latter class of biologically active factors constitutes the first step toward a better understanding of the mechanisms of life. This article deals with the latter category, which has been the major focus of our research in this area.

Although such "biological" assays monitor only for a specific activity, compounds thus isolated frequently exhibit other activities as well. Somewhat related is our finding (Kubo and Nakanishi 1977) that most compounds isolated from tropical plants as insect antifeedants showed activities other than disrupting the sense of taste of insects. For example, compounds isolated by an African armyworm antifeedant assay frequently turned out to be identical to those that had been isolated by cytotoxicity assays. Many of the insect antifeedants also turned out to be antibiotics. Thus, the antifeedant test provides a simple and convenient assay for isolating compounds having other activities as well.

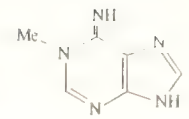
The topics described below have almost invariably represented a major challenge in isolation/bioassay, and for this reason many had eluded proper characterization. However, modern isolation and analytical techniques have enabled natural products chemists, with the assistance of scientists in other disciplines, to pursue projects that a decade ago would not have been feasible. It should be emphasized that isolation and characterization of a biologically active factor is but the beginning of a project, and not the end as is often considered. Once the structure of a bioactive factor has been determined, it becomes possible to plan projects that will clarify its mode of activity. Structure determination elevates a project to the level where we can start investigating the more dynamic aspects of life on a molecular structural basis, and in a manner less dependent on graphs and tables. This requires a multidisciplinary approach for which very few precedents, if any, exist. Needless to say, the factor should be synthesized if possible, to provide material for general screening as well as for mode of action studies.

Marine products probably should be processed differently from terrestrial products, but until further experience is gained most chemists are currently applying general terrestrial approaches to marine products. Several aspects of marine natural products make them inherently more difficult to process than terrestrial compounds: (a) If the isolation is from seawater itself, and as is frequently the case, the amount of the bioactive factor is miniscule, removal of salt presents a formidable problem. (b) Bioactive compounds transmitted through air can be collected by passing air through a column packed with absorbing material, but this is not possible in the case of seawater. (c) We are experienced in handling lipophilic compounds but many marine compounds tend to be hydrophilic. (d) Assays for specific biological activities in marine animals are difficult due to problems in mimicking the marine environment; furthermore, general knowledge regarding behavior of marine animals is very limited. The largely unexplored marine environment thus provides a promising, but challenging, source for new bioactive factors and biomedically useful compounds.

MEIOSIS-INDUCING SUBSTANCE, MIS

This work, carried out in collaboration with the late Professor H. Kanatani, then at the Ocean Research Institute, University of Tokyo, was our first entry into biologically active factors from marine sources (Kanatani et al. 1969). Studies of the spawning of sea stars, induced by injection of a water extract of radial nerves, showed that an active polypeptide (gonad stimulating factor, GSS) led to production of a meiosis-inducing substance (MIS) in the ovary (Fig. 1).

Ovaries of the sea star *Asterias amurensis* were placed in Petri dishes containing GSS-seawater (200 mg wet ovary per ml artificial seawater containing 200 μg lyophilized nerve) for 6 hr at 20°C. Meiosis-inducing activity was assayed by noting the breakdown of germinal vesicles of isolated oocytes of *A. pectinifera* after 1 hr. A total of 20 kg of ovaries were incubated in 100 l of GSS-seawater, the centrifugal supernatant was desalted, concentrated, and subjected to gel filtration (Sephadex G-15) and chromatography (CM-Sephadex C-25). In the late spawning season, this yielded 8.5 mg of MIS with meiosis-inducting activity at a concentration of 0.02 μg/ml. Spectroscopic studies characterized it to be 1-methyladenine, which was confirmed by synthesis. MIS is stable to treatment at 100°C for 30 min, and is species nonspecific. Determination of the structure of MIS and its ready availability by synthesis were critical factors



Meiosis inducing substance (MIS) from starfish *Asterial amurensis*

1) Gonad stimulating substance (GSS) from radial nerves produces MIS.

2) 200 mg wet ovaries / ml artificial seawater contng. 0.2 mg lyophil. nerve; 6 h. incubation

3) 20 kg fresh ovaries in 100 liters GSS-seawater

4) Gel filtration, Sephadex-CM gives 8.5 mg MIS (1-methyladenine).

5) Meiosis inducing activity: 0.02 microgram / ml.

Kanatani et al., Nature Vol.221, 273 (1969)

FIGURE 1. Meiosis-inducing substance.

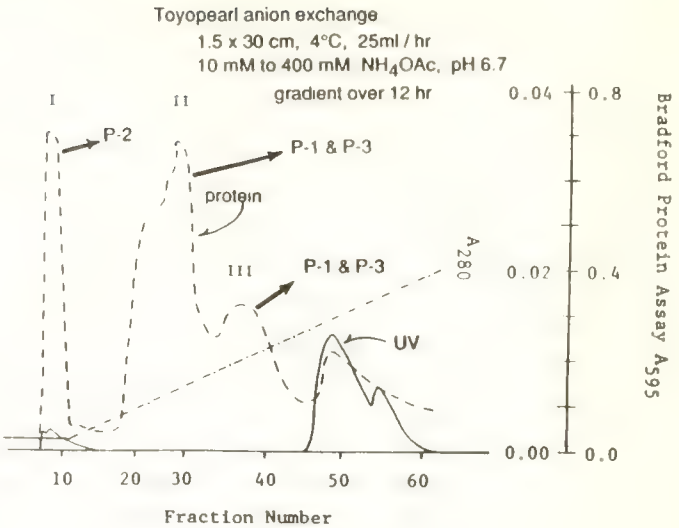


FIGURE 3. Chromatograph of pardaxins P-1, P-2, P-3.

leading to the elegant studies carried out by Kanatani, It is still the only meiosis-inducing substance characterized.

SHARK-REPELLING SAPONINS AND PEPTIDES FROM THE SOLE FISH

Upon disturbance, soles of the genus *Pardachirus* secrete defense toxins from the mucous glands lining their dorsal and anal fins. In particular, *P. marmoratus* (Red Sea Moses sole) has attracted attention as a shark repelling fish (Clark 1974). Primor et al. (1978) reported the isolation of the ichthyotoxic pardaxin ("protein") from its secretion, but its full primary structure is as yet unknown. Our investigation on the secretion from a congeneric sole, *P. pavoninus,* captured along sandy areas near coral reefs of Ishigaki Island, Ryukyu Archipelago, has resulted in the isolation and characterization of shark repelling ichthyotoxins, steroid monoglycosides (pavoninins-1 to -6) (Tachibana et al. 1984, 1985), and three peptides. Since these peptides are very similar to pardaxin they have been named pardaxins P-1 to -3, where P refers to the species name (Thompson et al. 1986).

Seven individuals of *P. pavoninus,* 20–30 cm long, were milked twice, one day apart, to give 30 g of lyophilized powder that yielded 10.7 g of a proteinaceous substance and 992 mg of saponins, both with strong detergent properties. Chromatography of the saponin mixture and monitoring by hemolytic activity and ichythyotoxicity (Japanese killifish) yielded the six pavoninins 1–6. Likewise, the secretion from *P. marmoratus* gave four similar saponins, mosesins 1–4 (Tachibana et al., unpublished). The structures of these lipophilic toxins are collectively shown in Figure 2. The A/B ring can either be *trans* or *cis,* the oxygen functions at C-3 can be 3α-OH, 3β-OH or 3-one, and double bonds may or may not be present at C-4/C-5/C-6; in all cases a sugar moiety is attached axially at 7α or 15α. As depicted in the conformational structure, the molecule has clearly defined hydrophobic and hydrophilic regions that give rise to its detergent and ichthyotoxic properties. It would be of interest to synthesize simpler steroid saponins (see Fig. 2) that might possess these attributes and test their pharmacological properties.

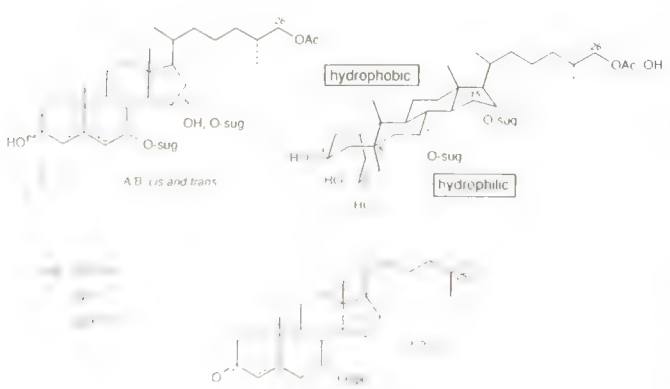


FIGURE 2. Shark-repelling saponins.

Typically 1 g of the lyophilized powder described above, in dilute acid or base treated with cold acetone, gave 420 mg of a precipitate free of pavoninins. The ichthyotoxic factor in the precipitate was first concentrated by gel filtration and then separated by anion exchange chromatography (Fig. 3, dotted chromatogram) into fractions I, II, and III by colorimetric protein assay (Bradford 1976). The major toxic fraction containing pardaxins P-1 and P-3 spanned a large volume of eluent that differed from run to run due to the strong surfactant nature of the chain molecules. Fractions II and III were often poorly resolved (Fig. 3), but occasionally they were base-line separated. Final purification was accomplished by HPLC; purity of the five components from the three fractions was demonstrated by SDS disc electrophoresis, which indicated the molecular weights of all peptides to be around 2,800.

HPLC retention times, amino acid composition, behavior toward enzymatic digestion, etc. all showed that the P-1 and P-3 peptides present in fractions II and III were identical; the peptides probably adopt multiple forms of aggregation with slow equilibrium, leading to their irreproducible separation on the anion-exchange column. It was subsequently found that separation of the peptides is greatly facilitated by chromatofocusing (Thompson et al. 1987). Amino acid sequencing clarified the

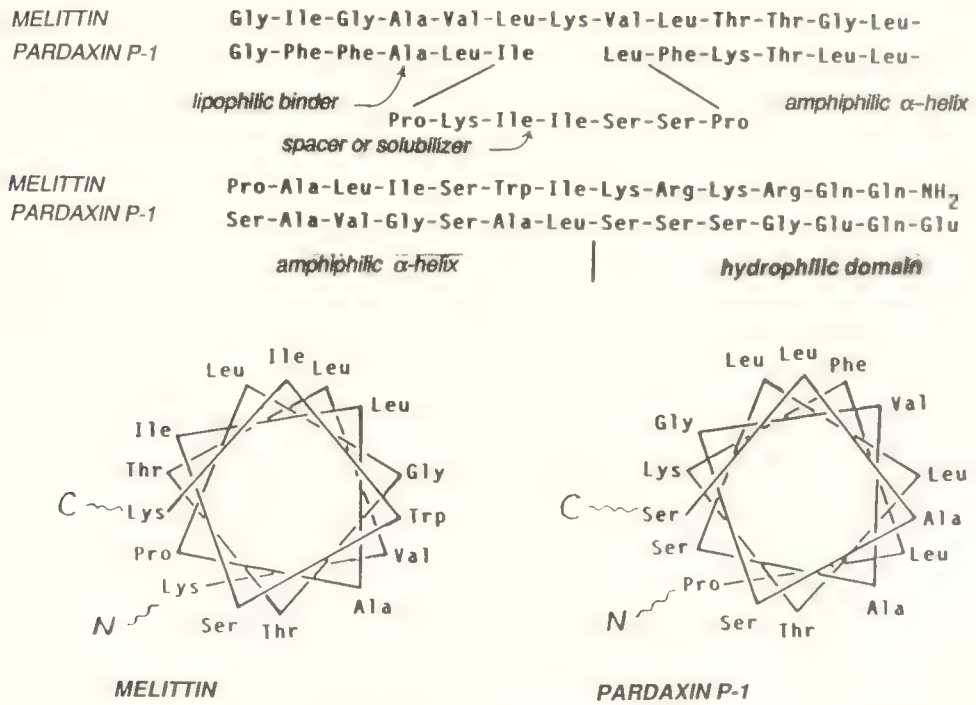


FIGURE 4. Comparison of melittin and pardaxin P-1.

primary structures of the three pardaxins (Thompson et al. 1986) (Fig. 4, pardaxin P-1). They are 33-peptides that consist of a lipophilic amino terminal and hydrophilic carboxyl terminal, and, similar to melittin (e.g., Dawson et al. 1978), the toxic bee peptide, the central section is capable of forming amphiphilic α helices. According to circular dichroic measurements, the pardaxins display α helical spectra in the presence of SDS (40% α helix), organic solvents or salts, but are largely random in water. Preliminary studies have demonstrated the qualitative similarities of the pardaxins to melittin: they lyse erythrocytes, display strong surfactant activity, and aggregate into tetrameric forms at high buffer concentrations. HPLC purification of pardaxins led to the isolation of two minor peptides that differed from the corresponding pardaxins only by the absence of the four amino acids from the hydrophobic amino terminal. Similar to melittin (Schröder et al. 1971), des-(1-4)-pardaxins are far less toxic than the pardaxins (<100-fold in rabbit erythrocyte hemolysis) and therefore the amino terminal must be involved in the binding to the membrane. With the syntheses of pardaxins P-1 and P-2 being achieved on an automated solid-phase synthesizer (Thompson et al. 1987), future studies are directed toward clarification of the mode of action. No region in melittin fits the sequence from Lys-8 to Pro-13 in P-1 (Fig. 4). In order to deduce the function of this fragment, des-(7-12)-pardaxin P-1 was synthesized; this led to marked insolubility, and not to an elevated hemolytic activity, which might have been expected on the basis of its apparently closer structure to melittin (Thompson et al. 1987).

BREVETOXINS, THE RED TIDE NEUROTOXINS

These toxins, found in the red tide dinoflagellate *Gymnodinium breve,* give rise to extensive fish kills along the Gulf of Mexico and Florida. Despite numerous attempts since 1968 to isolate the pure toxins, it was only in 1981 that two toxins, brevetoxins (BTX)-B and -C were isolated as crystals (Lin et al. 1981). The organism can be cultivated in the laboratory, a 20-l carboy culture yielding, after ether extraction and several flash chromatographs, 0.8 mg of BTX-A (noncrystalline, LD_{100} against the common zebra fish *Brachydanio rerio* 125 ng/ml), 5 mg of BTX-B (LD_{50} 16 ng/ml), and 0.4 mg of BTX-C (LD_{50} 60 ng/ml).

The structure of BTX-B (Fig. 5), determined by X-ray crystallography, disclosed an unprecedented molecule consisting of

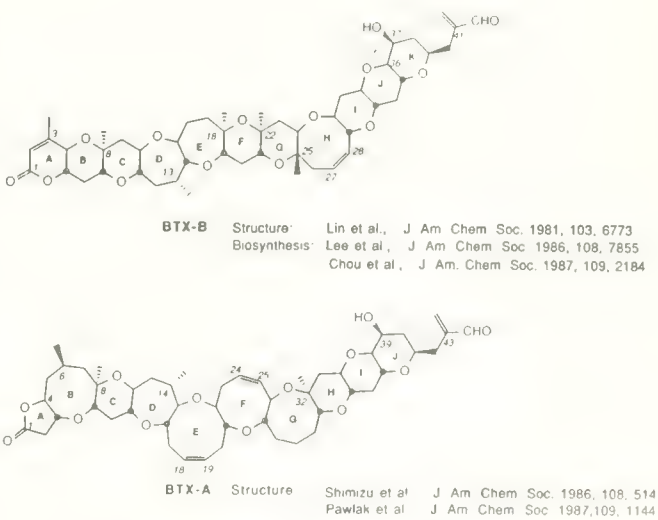

BTX-B Structure: Lin et al., J Am Chem Soc. 1981, 103, 6773
Biosynthesis: Lee et al., J Am Chem Soc 1986, 108, 7855
Chou et al., J Am. Chem Soc. 1987, 109, 2184
BTX-A Structure Shimizu et al J Am Chem Soc. 1986, 108, 514
Pawlak et al J Am Chem Soc 1987,109, 1144

FIGURE 5. Brevetoxin-B and brevetoxin-A.

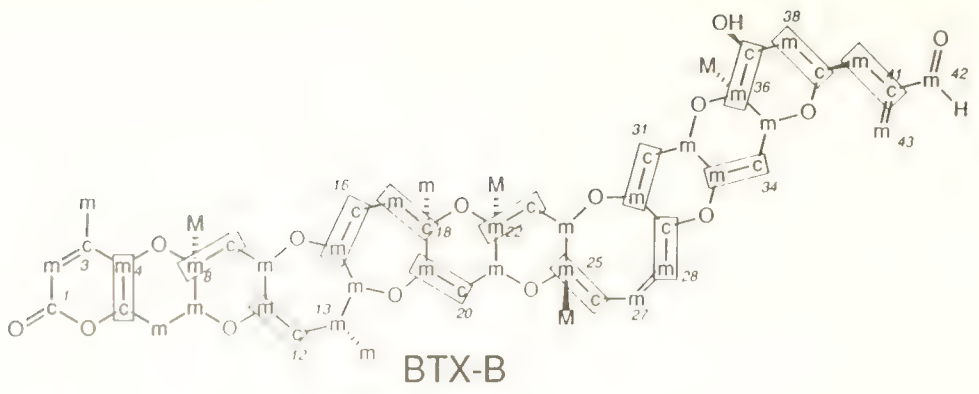


FIGURE 6. Origin of carbons in brevetoxin-B.

one lactone and 11 ether rings; the absolute configuration was determined by application of the exciton chirality method (Harada and Nakanishi 1972) on its derivative. Since all rings are *trans*-fused in a linear manner, the molecule, when constructed from models, resembles a stiff ladder with hinges at the junctions of the two seven-membered rings D and E. The 30 Å long BTX-B can bend to an angle of ca. 35°C at this juncture.

The brevetoxins act on the voltage-dependent Na^+ channel (Huang et al. 1984; Catterall and Gainer 1985; Wu et al. 1985), but intriguing aspects such as structure-activity relations of this long lipophilic molecule are not known. Preliminary studies carried out with G. Strichartz (unpublished) have shown that reduction of the double bond or the C-41 aldehyde increases the activity (measured by depolarization of frog sciatic nerve) three- to seven-fold, but hydrogenation of the ring A double bond reduces the activity by almost 10-fold. Photoaffinity labeled BTX-B carrying a diazoacetate group at C-42 has been prepared (Lee, unpublished) and will be employed to study the mode of binding, etc. The unprecedented structure also involves a puzzling biosynthetic scheme that is under investigation (Lee

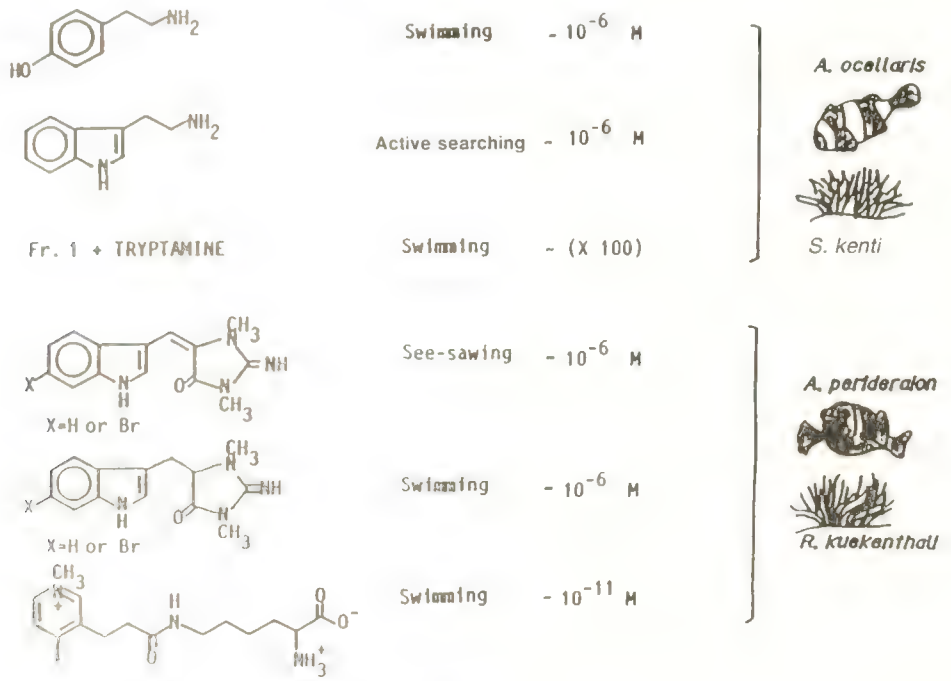
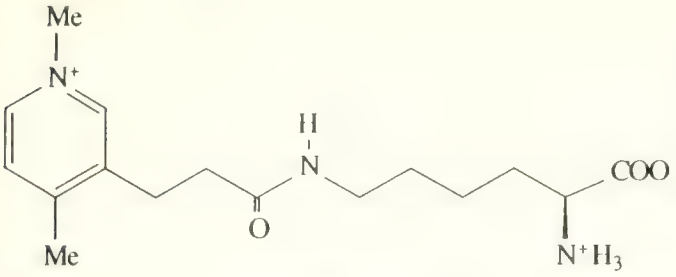


FIGURE 7. Species-specific synomones.



1 Amphikuemine

et al. 1986; Chou and Shimizu 1987). As shown in Figure 6 (Lee et al. 1986; Chou and Shimizu 1987), where "m" denotes carbons originating from the methyl group of acetic acid, several contiguous m-m and m-m-m moieties, and even an m-m-m-m group, have been clarified by incorporation studies with ^{13}C precursors.

Shimizu and co-workers (1986) recently succeeded in crystallizing BTX-A, the most toxic of the brevetoxins, and this led to the X-ray derived structure shown in Figure 5. An exhaustive NMR and mass spectroscopic (MS) study carried out on two derivatives of BTX-A, $C_{49}H_{70}O_{13}$, allowed us to arrive independently at the same structure except for the 6-methyl configuration, which was deduced as being α rather than β. This is corrected in the full account (Pawlak et al. 1987). During the course of these studies, two general fragmentation patterns in the MS of brevetoxins derivatives were recognized, and it was these patterns, assisted by NMR data, that enabled us to reconstruct the structure of BTX-A in a logical manner. The BTX-A skeleton represents another extraordinary structure consisting of *trans*-fused 5/8/6/7/9/8/8/6/6/6 lactone-ether rings. Its different ring structure makes it far more wobbly than BTX-B, hence reducing its crystallinity and leading to broadened [^{1}H]-NMR signals.

AMPHIKUEMINE, A POTENT SYNOMONE

Sea anemones, like all cnidarians, produce peptidic toxins that are used both offensively and defensively (Schweitz et al. 1985). Certain sea anemone species maintain a symbiosis with anemonefishes that are protected from these peptides by a mucous coat (Miyagawa 1983). "Synomones" (Nordland and Lewis 1976) are chemicals that induce such a symbiosis. Miyagawa (1983) demonstrated that four species of anemonefishes, including *Amphiprion perideraion,* which is symbiotic with "*Radianthus kuekenthali*" (the correct name for which is *Heteractis crispa* [see Dunn 1981]) and *A. ocellaris,* which is symbiotic with "*Stoichactis kenti*" (properly *Stichodactyla gigantea* [see Dunn 1981]) (Fig. 7) are attracted to their specific partner because of chemicals secreted by the anemone. After the 7–10 day planktonic period that follows hatching, juvenile fish settle into a partner sea anemone, beginning the symbiosis. As depicted in Figure 8, visual cues do not play an important role in recognition of the host, the fish being indifferent to their symbiotic anemone if the host is placed in a second transparent vessel in the same tank. However, they are attracted to seawater originating from the second vessel (even a juvenile that had not previously encountered one of its specific partner anemones).

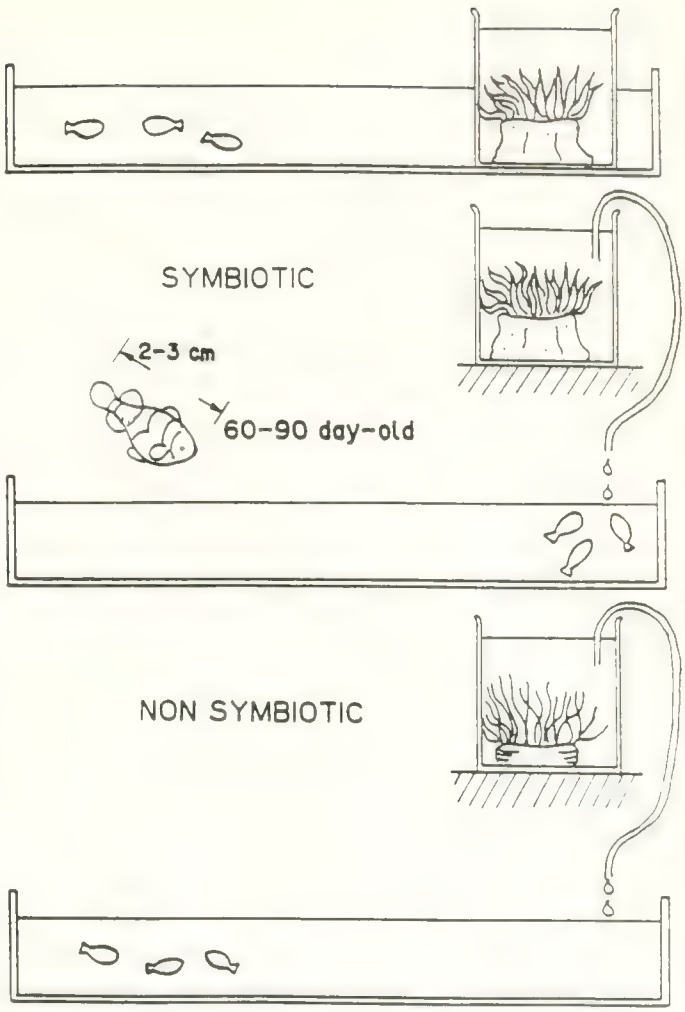


FIGURE 8. Response of anemone fish to sea anemone.

However, the fish behaves indifferently if the anemone is not of a species with which the fish is normally symbiotic (bottom of Fig. 8).

A compound "amphikuemin" **1** (from *Amphiprion* and *kuekenthali*), which induces characteristic attracted swimming at a concentration of 10^{-11} M, and several other chemicals that elicit characteristic symbiotic movements have been identified for the first time (Murata et al. 1986) (Fig. 7). Ten specimens of *H. crispa* (15 kg) collected off Sesoko Island near the Okinawa Expo Memorial Park Aquarium were homogenized, and the 1% acetic acid/20% aqueous methanol extract was passed through a series of chromatographic columns as monitored by an attracted swimming assay. This yielded 48 μg of a cationic compound as the active factor, the induced behavioral response being comparable to that induced by the crude extract. Structure **1** was determined by spectroscopic methods and hydrolysis that gave L-lysine (Murata et al. 1986), and has been verified by synthesis (Konno et al., unpublished). A total of 16 aplysinopsins and dihydroaplysinopsins, which induce "see-sawing" (up-and-down head movement) and swimming, respectively, were also isolated, but the effective dose is much larger and their real role in symbiosis is not clear.

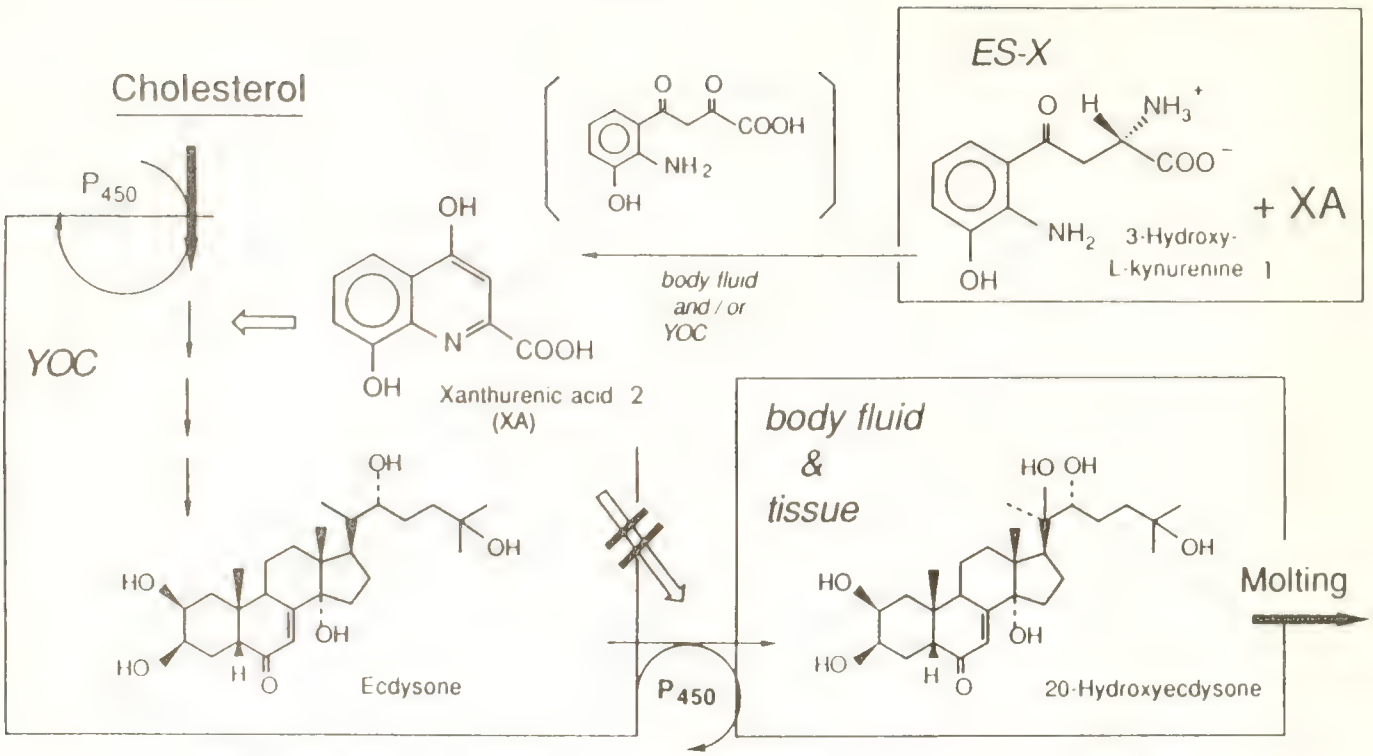


FIGURE 9 Relation between ecdysone biosynthesis and inhibitor. "ES-X" corresponds to that shown in Figure 10.

The pair *A. ocellaris* and *S. gigantea* similarly led to the characterization of tyramine and tryptamine, which induced attracted swimming with tail wagging and active searching behavior, respectively, both at a dose of 10^{-6} M. However, since the potent attracted swimming activity of the crude extract of the anemone could not be reproduced by a single pure fraction, we conclude that the synomonal activity of the secretion is caused by tyramine together with the synergistic effect of tryptamine and several unidentified chemicals.

Interestingly, tyramine and tryptamine are both neurotransmitters. Currently amphikuemine is being synthesized in larger quantities to test for neurobiological or any other pharmacological effects. The quaternary pyridinium structure of amphikuemine plays an important role because desmethyl-amphikuemine, lacking the methyl group, is inactive; presumably, the positive charge increases the affinity of amphikuemine to the negatively charged membrane surface.

ENDOGENOUS INHIBITOR OF ECDYSONE BIOSYNTHESIS IN CRABS

The life cycle of crustaceans involves periodic shedding of the exoskeleton. This is controlled by 20-hydroxyecdysone (Fig. 9), the common molting hormone of crustaceans (and all insects), which is synthesized in the Y-organ located in the thorax (Skinner 1985), and a molt-inhibiting hormone (MIH). The latter is produced in the X-organ located in the eye stalk (ES), and controls the biosynthesis or release of ecdysone (Chang and O'Connor 1977; Jegla et al. 1983). It has been known since the early 20th century that removal of ES promotes molting and premature ecdysis, whereas implantation of ES reverses this effect (Zeleny 1905). It was shown by *in vitro* experiments that crab tissues, especially the testis but not the Y-organs, are capable of hydroxylating ecdysone into 20-hydroxyexdysone (Lachaise and Feyereisin 1976; Chang and O'Connor 1978).

Evidence was first obtained in 1982 that sinus gland extract of the crab *Pachygrapsus crassipes* decreases the titer of circulating ecdysteroids (Keller and O'Connor 1982). Recently several peptides with MIH activity have been isolated from the sinus glands of crustaceans, i.e., a peptide with molecular weight 6,000–14,000 from the crab *Carcinus maenas* (see Webster and Keller 1986), and two closely related peptides with molecular weight ca. 8,700 from the lobster *Homarus americanus* (see Chang et al. 1987). On the other hand, there is also evidence that the ES of the shrimp *Pandalus jordani* contains a small molecule that is involved in molt inhibition (Soyez and Kleinholz 1977). Possibly the former is a hormonal releasing factor or neurotransmitter leading to the release of MIH. Recently the MIH activity of 5-hydroxytryptamine on the isolated crab eyestalk ganglion has been reported (Mattson and Spaziani 1985).

The isolation (Fig. 10) and characterization of a species-nonspecific compound with MIH activity on ecdysone biosynthesis is outlined in the following (Naya et al., submitted). *Callinectes sapidus* (blue crab) and other crab species (a mixture of sexes, maturity, and molt stages) were collected off various coasts of U.S.A. and Japan. After crabs were immobilized by chilling on ice, their ES were excised, frozen with dry-ice, and lyophilized to prevent deterioration of the tissue during storage at −5°C as the "MIH pool." The destalked crabs were killed by acute freezing with liquid nitrogen, then stored at −80°C until their Y-organs were excised.

The protocol for bioassaying the MIH activity of the X-organ

extract is outlined in Figure 10. After thawing, the Y-organ and adhering tissues ("Y-organ complex" or YOC) were dissected, washed with buffer to remove the "MIH" adhering to the tissues, and homogenized to the chilled buffer. The suspension was centrifuged, and aliquots of 2.5 ml/Y-organ were incubated at 37°C with shaking for 20 hr. After incubation, each aliquot was lyophilized, was extracted with MeOH, and the extract was submitted to HPLC analysis for ecdysone quantification. The amount of ecdysone in the cultured homogenates was usually 5–10 ng/Y-organ. The ES stored as the "MIH pool," 660 organs from 330 crabs weighing ca. 36 g each, was extracted with 0.1 M acetic acid at 100°C for 10 min (the MIH factor is thermally stable), the extract was centrifuged, and the supernatant was lyophilized and stored as the crude ES extract ("ES-X"). The inhibitory action of the extract of ecdysone biosynthesis was assayed by incubation with the YOC homogenate in an ES/Y-organ ratio of 1:1 and measurement of the reduction in HPLC peak area. Isolation of the "MIH" from 36 g of ES following this protocol led to the isolation of 700 μg of a single compound which was identified as 3-hydroxy-L-kynurenine **1** (3-OH-K, Fig. 9). Incubation of YOC homogenate with authentic 3-OH-K reproduced the MIH activity; however, the potency was less than that of the crude ES extract.

When xanthurenic acid **2** (Fig. 9), which together with 3-OH-K is a key metabolite of tryptophan, was tested for MIH activity, surprisingly it was found to be stronger than 3-OH-K. An active search for **2** itself then led to its detection in ES-X, the estimated amounts of **1** and **2** in the crude ES extract being 3.4 μg and 2.0 μg/ES, respectively. However, HPLC analysis showed that the contents of **1** and **2** in the carefully collected X-organ/sinus gland complex were 28 ng and 128 ng/ES, or 1% and 7% of the content in ES-X, respectively.

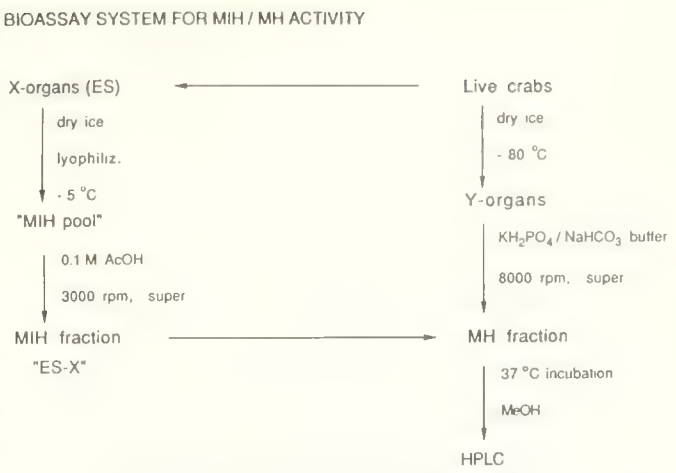


FIGURE 10. Bioassay system for MIH/MH activity.

A crude enzyme preparation from the crab body fluid led to a 60% conversion of **1** into **2** after a 3 hr 37°C incubation. It is conceivable that 3-OH-K is transported from the X-organ to the Y-organ and converted into xanthurenic acid in the body fluid of YOC; the YOC homogenate before incubation often contains some **2** (ca. 350 ng) but little (ca. 35 ng) or none of **1**. Preliminary studies suggest that **2** exerts its ecdysone biosynthesis inhibitory action by inactivation of the cytochrome P-450 hydroxylation system (Miki et al., unpublished). The body fluid contains ecdysone-20-hydroxylase, as shown by an ecdysone to 20-hydroxyecdysone conversion; however, **2** does not suppress this 20-hydroxylation in the body fluid (unpublished).

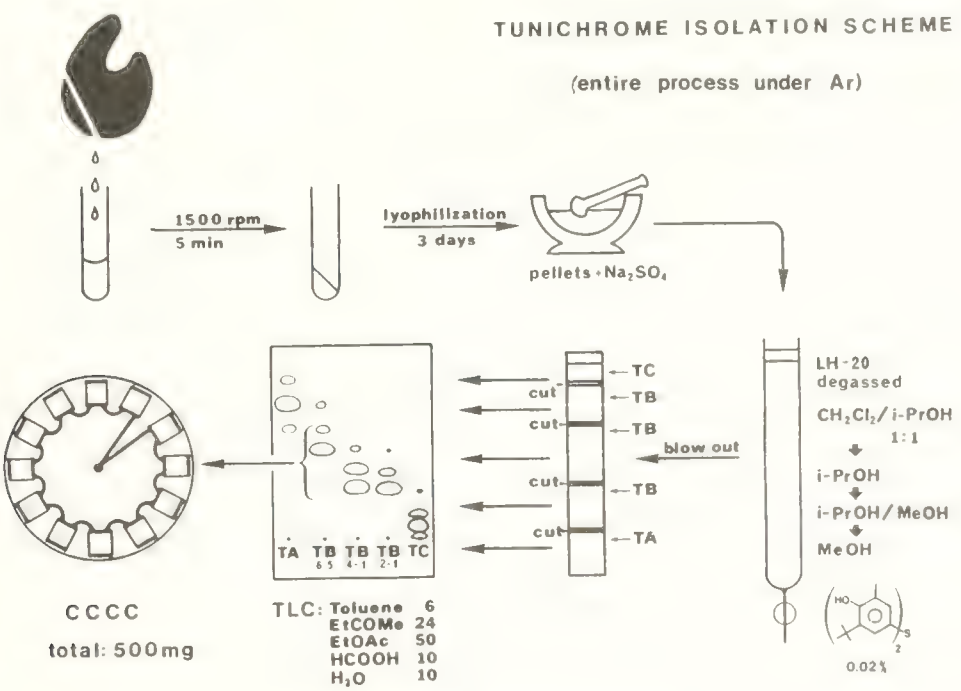


FIGURE 11. Isolation scheme of tunichromes.

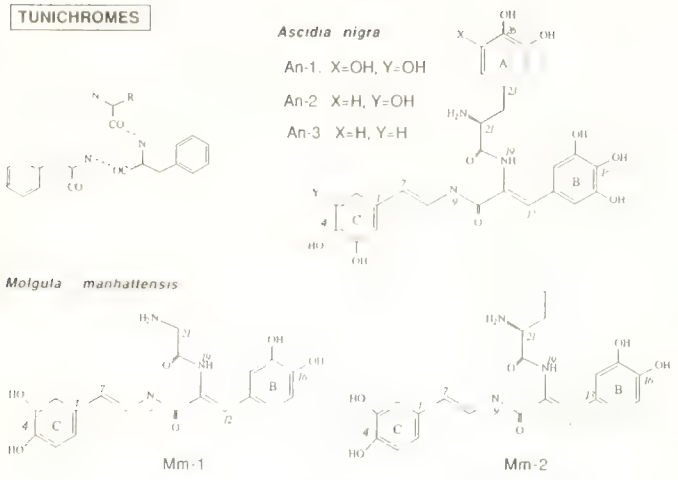


FIGURE 12. Various tunichromes.

TUNICHROMES, REDUCING BLOOD PIGMENTS FROM TUNICATES

The tunicates, or sea squirts, are common marine organisms distributed throughout the world, with approximately 2,000 known species. Some are solitary, others form colonies. Some species, especially the orange-red *Halocynthia roretzi* that is distributed widely in Japan, are treasured as an appetizer and eaten raw by some people (including this author). The hematology of tunicates has puzzled scientists for over 70 years since Henze (1911) discovered that the blood of *Phallusia* (*Ascidia*) *mammillata* contains large quantities of vanadium, the blood pH is below 2, its color in air changes from yellow-green to red-brown to dark blue, and addition of barium ion gives rise to a white precipitate. The precipitate was erroneously believed to be barium sulfate and hence the acidity was ascribed to sulfuric acid, but it is now known that the precipitate is barium vanadate. The pH within the blood cells is still a matter of controversy. Tunicates of other species accumulate Fe, Mo, and Nb, rather than V.

The extreme sensitivity of the blood pigment to air had eluded all isolation attempts. After numerous failures over a period of five years, we arrived at the scheme shown in Figure 11 that resulted in the structure determination of this new group of blood pigments (Bruening et al. 1985, 1986). The entire process is carried out under deoxygenated Ar, with exclusion of moisture. The tunicate *Ascidia nigra* was brought from Florida alive in lots of 1,000, and immediately processed. Collection of blood from 1,000 animals is a five hour process carried out by 10 people. The isolation was probably the most difficult procedure carried out in our laboratory because of the extreme sensitivity of the pigments (tunichromes). Tunichromes readily decompose on HPLC, but luckily a prototype centrifugal counter-current chromatograph (CCCC, now called centrifugal partition chromatograph or CPC) became available in our laboratory. Only through this method was the semipreparative scale purification after the LH-20 step achieved. The final purification for structural studies had to be performed by HPLC despite 90% loss in material upon one passage. Shaving the closely eluting HPLC peaks of a 5 mg mixture of tunichrome B-1 and B-2 (since renamed An-1 and An-2, "An" representing *A. nigra*) yielded 500 μg pure TB-1 (An-1) for the first time. This corresponds to a yield of 18 mg of An-1 from 1,000 tunicates.

Subsequently, two other tunichromes—An-2 and -3—have been identified (Fig. 12). Moreover, the blood of the iron-accumulating *Molgula manhattensis* has been found to contain pigments that lack one of the phenolic rings (Oltz, unpublished). The structures of these tunichromes suggest that they are biosynthesized from the condensation of three amino acids in the manner shown in Figure 12.

Although the structures of several of these new blood pigments have been elucidated, it merely represents the beginning of a series of far more complex and interdisciplinary studies, some of which are being carried out in our laboratory. What is the biological role of tunichromes? What is the relation between these pigments and the metal? Why are different metals accumulated by different species of tunicates? How does the metal exist within the blood, and what is its valency? The tunicates contain several different types of blood cells, but are the metal and tunichromes contained in different types of cells? (Preliminary experiments with fluorescence activated cell sorter suggests that this is the case; however, a tunichrome/metal complex may be present in some cells.) Answers to some of these questions will clarify the biochemical role of vanadium in mammalians as well.

DISCUSSION

In any study in the area of modern natural products chemistry, particularly when dealing with "biological factors" as exemplified above, isolation and purification are the mandatory first steps to accomplish. Unfortunately, this phase is often handled too casually, without the realization that success or failure of a project may be determined by this first step. Even when an air-sensitive compound is isolated by ingenious manipulations, or a hormone is isolated in miniscule amounts from tons of starting material and after years of frustrating bioassays, the purification protocol is normally applicable only to that particular case and lacks generality. The purification process is usually presented at symposia in one to two slides and is seldom followed by discussion. It may even be said that the most exciting problems in characterization of the "biological factor" intimately related to the maintenance of life are those that address the challenge of compound isolation. Difficulties include miniscule quantities, sensitivity to air/light/moisture etc., difficulty in assay, a compound's transient existence, or inherent difficulties such as sticky detergents or complex mixtures of oligomers.

In the majority of cases, structure determination is far less challenging than isolation. Characterization of a natural product and its synthesis used to be the ultimate objective of a chemist in this area. Now, in addition to exploring possible applications of the bioactive compound in biomedical areas, the most challenging problem is to clarify its mode of action on a concrete structural basis. This involves understanding the interaction of the factor with its receptor molecule and the subsequent cascade of conformational and/or structural changes of numerous other molecular species. Such studies, which have become conceivable only during the past few years, clearly require a multidisciplinary approach encompassing all areas of science.

ACKNOWLEDGMENT

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Peptide Chain Toxins of Marine Animals

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INTRODUCTION

Peptide chains (peptides, polypeptides, and proteins) are possibly the most common pharmacologically-active substances in marine animals. Until recently they attracted little interest from natural products chemists, since their isolation and chemical characterization often required different techniques than for smaller, more lipophilic molecules. Synthesis was practical only for peptides and small polypeptides. However, recent technical innovations in the purification, sequence determination, synthesis, and higher structure analysis of proteins have greatly facilitated the investigation of these substances, even when they occur in only trace amounts. Once the sequence of a peptide chain toxin is known, solid-phase chemical synthesis or recombinant DNA methods can be exploited to produce larger quantities of the substance (and analogs) for research, therapy, and other applications. Crystallographic and nuclear magnetic resonance spectroscopic techniques can determine the influence of single amino acid substitutions upon tertiary structure. This "protein engineering" approach should permit structural manipulation of therapeutically promising peptide chain toxins, and may eventually allow the design of smaller, more bioavailable, less toxic analogs of these substances.

This paper surveys present knowledge concerning the chemistry of 37 distinct types of peptide chain toxins so far isolated from marine animals. Particular attention is focused on neurotoxins of sea anemones, nemertine worms, and gastropods, which have been most extensively investigated. These three animal groups produce a variety of toxins that selectively affect membrane ion channels involved in the function of the nervous, cardiovascular, and muscular systems.

ISOLATION AND CHARACTERIZATION OF PEPTIDE CHAIN TOXINS

The investigation of a peptide chain toxin may be conveniently divided into several stages, as shown in Figure 1. Unless one is concerned only about the public health aspects of intoxication or envenomation, it is clearly necessary to purify individual active constituents of an extract or venom before their mechanisms of action can be investigated properly. A venom is a secretion that usually contains several biologically-active substances, while a toxin may be defined as a single substance with poisonous or otherwise deleterious properties. Whether used as a research tool or as a therapeutic agent, most venom constituents are generally used separately; therefore most investigations attempt to characterize the properties of individual toxins.

The initial collection, identification, and extraction of biologically-active substances from marine organisms are extremely critical steps in their investigation. It should not be necessary to stress the importance of proper identification of the organism utilized. However, recent investigations of sea anemone toxins demonstrate some of the problems resulting from ignoring the importance of sound taxonomy. One problem has been the primitive state of anthozoan systematics. Referring to a sea anemone toxin source by a scientific name is not satisfactory by itself, since some species have been referred to by many different scientific names and a single name may have been applied to more than one species. It is thus very important to preserve voucher specimens and, if possible, to provide written and photographic descriptions of an organism in its living state (Dunn 1981).

Without knowing the concentration of a toxin present in an organism, there is always some uncertainty regarding how many animals should be collected, particularly if the locale is not readily accessible. In this situation, one should avoid overcollecting to the detriment of the animal population, particularly because modern analytical methods for investigating proteins are much more sensitive than they were a decade ago. Since smaller amounts of tissue now usually suffice for analysis, one can usually avoid mixing specimens from geographically separate populations which may possess different peptide variants.

Active proteins are to be initially extracted from an organism in a highly enriched form if at all possible. Even if overall yield is reduced, it is often better to use only tissues or secretions in which the toxins are localized. This toxin-enriching "biological" purification step also usually reduces the likelihood of proteolytic degradation, which can also be minimized further by expeditiously working up extracts at low temperature in the presence of suitable protease inhibitors.

An incredible variety of separation methods is now available for purifying proteins, whatever their source. High pressure liquid chromatograph supports of various types (including weakly acidic and basic ion exchangers for proteins) have permitted much better separations than previously. For instance, Wachter et al. (in press) recently isolated nine different polypeptide variants from the sea anemone *Anemonia sulcata* using reversed phase HPLC, whereas only three variants had been previously separated. Isoelectric focusing also is an extremely useful analytical and preparative method since it is capable of separating proteins with only small (0.05) pI differences. The most unique feature of a protein is its surface topography, including receptor binding domains and antigenic sites. Immunoadsorbent chromatography with monoclonal antibodies is thus an extremely powerful means of protein isolation. So far, the only example of its application to a marine natural product has been the partial purification of a jellyfish toxin (Cobbs et al. 1983).

No single method for evaluating protein purity can be relied upon entirely. Unfortunately, many investigators have placed too much faith upon single bands in SDS gels as proof of homogeneity. Since the most difficult problem in most protein purifications is separating homologous (isoenzymes, isotoxins, etc.) variants differing in only a few amino acid residue substitutions, this method clearly can be misleading when used alone.

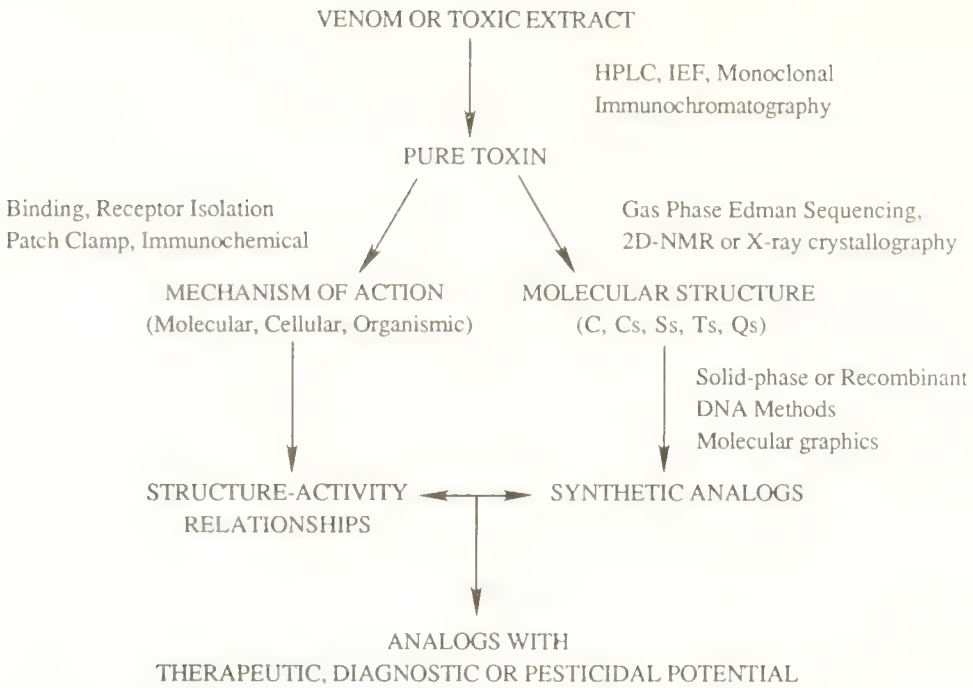

FIGURE 1. The major stages of peptide chain (peptide, polypeptide, or protein) toxin investigation. Some recently introduced methodologies are noted. Abbreviations: Co, amino acid composition; Cs, covalent structure; Ss, secondary structure; Ts, tertiary structure; Qs, quaternary structure.

The best methods for evaluating purity besides SDS gels are probably isoelectric focusing, reversed phase HPLC, immunodiffusion or immunoelectrophoresis, and N-terminal sequencing.

Elucidation of a protein structure usually begins with amino acid analyses and then proceeds to Edman sequencing. With the advent of the gas phase sequencer, it is now possible to sequence perhaps a nanomole of protein for 40–50 steps. New mass spectrometric techniques including fast atom bombardment, tandem mass, and plasma desorption are increasingly able to give meaningful structural information on even large (25,000 daltons) peptide chains. One important advantage of mass spectrometry is its ability to analyze an unresolved mixture of peptides (Delgass and Cooks 1987).

Determining the amino acid sequence of a large protein (>20,000 daltons) can be a time-consuming and expensive venture using the Edman degradative strategy, since many peptides have to be isolated and individually sequenced. In the future, the sequencing of cloned cDNA will probably become the preferred method for investigating many of the large proteins listed in Table 1, due to its speed, low cost, and high sensitivity.

Obtaining the covalent structure of a protein is more a means to an end than an end in itself. However, it does permit: (1) chemical or recombinant DNA synthesis, (2) predictions of secondary structure, and (3) rigorously controlled chemical modification studies. Ten years ago circular dichroism spectroscopy was the only means of measuring secondary structure other than by x-ray crystallography. Now it is also possible to utilize laser Raman and two-dimensional NMR spectroscopic methods to obtain this information. The elegance of 2D-NMR is that it also locates the secondary structure within the sequence (however, this technique is currently limited to small proteins). Although x-ray crystallography yields the most precise tertiary structural details, some proteins and peptides cannot be readily crystallized. Now 2D-NMR, in conjunction with distance geometry algorithms, can provide fairly precise tertiary structures for small proteins and peptides (Kobayashi et al. 1985). Once a tertiary structure becomes available it is possible to visualize the molecule's surface using molecular graphics computer software. This facilitates the rational planning of chemical modification and synthesis experiments to determine the functionality of particular regions on the surface of the molecule.

Several major innovations have also occurred in recent years that greatly facilitate physiological and pharmacological investigations of the mechanisms of action of protein toxins. Toxins affecting ion channels can now be investigated at the single channel level by means of the patch clamp technique pioneered by Neher and Sakmann (1976). This method (as well as the earlier artificial bilayer method) also should be useful for investigating the mechanisms by which certain protein cytolysins (*Stichodactyla* toxin and other channel-formers) permeabilize their target cell membranes. Another major development has been in the analysis of drug and toxin binding to membrane receptors using radiolabelled ligands. It is now possible to determine whether two toxins bind to the same or to different sites even if they produce the same effect, without recourse to less quantitative physiological experiments comparing the effects of mixtures of the substances with the effects of each isolated substance.

Table 1 lists the various distinct types of linear peptide, polypeptide, and protein toxins so far detected in marine animals. Since there is little concensus regarding the definitions of "peptide," "polypeptide," and "protein," I shall frequently refer to the toxins collectively as "peptide chain" toxins. The only prerequisite for inclusion of a toxin in this table was an approximate molecular size and evidence that the biological activity detected was related to a particular gel or ion exchange column eluant zone. In many cases other variants (isotoxins) have also been

Table 1. Toxic Peptides, Polypeptides, and Proteins Isolated from Marine Animals.

Animal Source	Toxin Characteristics: Primary Action[1]	Molecular Size[2]	Structural Analysis[3]	Investigators
Spongifera				
Suberites (Sea Orange)	C	28,000	Co	Cariello et al. (1980)
Cnidaria				
Physalia (Man O'War)	C,I	240,000	-----	Tamkun and Hessinger (1981)
Millepora (Fire Coral)	C,I	100,000	-----	Wittle et al. (1971)
Chironex (Box Jellyfish)	ME,I	150,000	-----	Crone and Keene (1969)
	C,I	70,000	-----	Crone and Keene (1969)
Cyanea (Lion's Mane Jellyfish)	ME,I	70,000	-----	Walker (1977)
Aiptasia (Acontiate Anemone)	C	135,000		Hessinger and Lenhoff (1976)
	P	43,000		Grotondorst (1979)
	C	12,000		Hessinger and Lenhoff (1976)
	N	9,000		Hessinger et al. (1973)
Metridium (Frilled Anemone)	C	~80,000	-----	Bernheimer and Avigad (1978)
Stichodactyla (Sun Anemone)	C,I	17,000	Co,Cs,Ss	Blumenthal and Kem (1983)
	N(Na)	5,000	Co,Cs,Ss	Kem et al. (1986)
Anemonia (Waxrose Anemone)	N(Na)	5,000	Co,Cs,Ss	Béress et al. (1975)
	N(Na)	3,000	Co,Cs,Ss	Béress et al. (1977)
Goniopora (Coral)	N(Na)	10,000	-----	Gonoi et al. (1986)
Nemertinea				
Cerebratulus (Milky Nemertine)	C	>30,000	-----	Kem and Blumenthal (1978)
	C	11,000	Co,Cs,Ss	Kem and Blumenthal (1978)
	N(Na)	6,000	Co,Cs,Ss	Kem (1976)
Lineus (Red Ribbon Worm)	N(Na)	6,000	-----	Kem (1973)
Annelida				
Glycera (Blood Worm)	N	300,000	-----	Bon et al. (1985)
Mollusca				
Aplysia (Sea Slug)	C,I	45,000	-----	Merker and Levine (1986)
Conus (Cone Shell)	N(Na)	28,000	-----	Kobayashi et al. (1982)
	N(K)	>10,000	-----	Chesnut et al. (1987)
	N(Na)-μ	2,000	Co,Cs	Sato et al. (1983)
	N(Ca)-w	3,000	Co,Cs	Olivera et al. (1984)
	N(ACh R)-a	1,000	Co,Cs,Ts	Gray et al. (1981)
Octopus (Pacific Octopus)	N	23,000	Co	Songdahl and Shapiro (1974)
Eledone (Octopus)	N	1,000	Co,Cs	Erspamer and Anastasi (1962)
Loligo (Squid)	C	~40,000	-----	Kem and Scott (1980)
Echinodermata				
Tripneustes (Sea Urchin)	I	>70,000	-----	Feigen et al. (1970)
	N	25,000	-----	Mebs (1984)
Toxopneustes (Sea Urchin)	I	20,000	-----	Nakagawa and Kimura (1985)
Lytechinus (Sea Urchin)	N	5,000	-----	Kem (in preparation)
Chordata				
Scorpaena (Scorpion Fish)	N	>50,000	-----	Schaeffer et al. (1971)
Laticauda (Sea Snake)	N	7,000	Cs,Ss,Ts	Low et al. (1976)
Pognoperca (Soapfish)	C	4,000	Co	Hashimoto (1979)
Pardachirus (Red Sea Sole)	C	3,000	Cs	Thompson et al. (1986)

1. Abbreviations: C, cytolytic; I, inflammatory; ME, excitatory (depolarizing); N, neurotoxic (Na, sodium ion channel; Ca, calcium ion channel; K, potassium ion channel; ACh R, acetylcholine (nicotine)-activated channel); P, phospho lipase.

2. The molecular size estimates are,in many instances, apparent molecular sizes based upon chromatographic elution behavior of the undenatured toxin; they are rounded off to the nearest 1,000 daltons.

3. Abbreviations: Co, amino acid composition; Cs, covalent structure; Ss, secondary structure; Ts, tertiary structure.

characterized, but were not included for the sake of brevity. The designation of a primary type of action has been admittedly arbitrary and speculative in some cases where little information is yet available, but is meant to indicate to the reader the most probable site of action of the toxin. A neurotoxin is defined as a substance that adversely affects neuronal and/or muscle cells, but not other cells. A membrane-excitatory toxin is considered a toxin that at least initially causes depolarization or some other form of stimulation to various types of cells besides those of nervous and muscular tissues. An inflammatory toxin is defined as a substance that primarily causes inflammation with little or no direct neurotoxic or cytolytic effects. Cytolysins are perhaps the most arbitrarily defined toxins since probably few of these substances produce their characteristic symptoms in envenomated animals by lysing cells. This term is widely used to refer to substances capable of causing cell lysis under *in vitro* conditions.

NEUROTOXINS

Cnidarian Neurotoxins

The phylum Cnidaria certainly contains the greatest number of toxic species of any animal phylum. Over 10,000 species have been described. Every group probably possesses toxins, since nematocysts (cnidae) are ubiquitous. One could argue that polypeptides and proteins must be the only toxic constituents of these stinging capsules, since it has been shown (Lubbock and Amos 1981) that the proteinaceous nematocyst wall is permeable to solutes of 600 daltons or less. However, it is possible that smaller molecules could be immobilized (bound) within the nematocyst capsule, or that the Golgi-type membrane surrounding the nematocyst actively transports small toxins into the capsule. It would be interesting to determine whether the terpenes of soft corals are localized within nematocysts.

There are four classes of cnidarians: (1) Hydrozoa, (including the Man O'War, *Physalia*), (2) Scyphozoa (jellyfish), (3) Cubozoa (boxjellies), and (4) Anthozoa (soft corals, octocorals, and sea anemones). Richet (1903) initiated the study of cnidarian venoms and at the same time serendipitously discovered anaphylaxis while investigating the toxicity of *Physalia* tentacle extracts upon dogs. Without chromatographic and other modern separation techniques, he demonstrated the presence of three different types of biologically active substances in sea anemone (*Actinia* and *Anemonia*) extracts: (1) water-soluble neurotoxins, (2) "congestine," a less water soluble constituent causing pulmonary edema, and (3) "thallasine," an ethanol soluble constituent causing inflammation and hypotension.

At present, only the Anthozoa has been shown to possess toxins (peptide and otherwise) that specifically interfere with the function of nervous or muscular tissue. Three different molecular size classes of polypeptide neurotoxins have so far been isolated from this group and it will be surprising if others are not found as the class is more extensively explored. These polypeptides all seem to act in a similar fashion when studied electrophysiologically: they prolong the repolarization phase of sodium channel-mediated action potentials by slowing the rate of Na channel inactivation. The result for the envenomated organism is quite serious, since the normally millisecond duration action potential may now last as much as a second after the toxin has its effect. This results in a massive release of neurotransmitters at nerve terminals, causing hyperexcitability, convulsions, and often death.

The first polypeptides were isolated by Béress et al. (1975) from the waxrose anemone, *Anemonia sulcata*. As-I (highly toxic to arthropods) and As-II (highly toxic to vertebrates) are 5,000 dalton single polypeptides containing three disulfide crosslinks (Wunderer et al. 1976). As-III is a 3,000 dalton polypeptide possessing four disulfide crosslinks. During the past decade, several other anemone polypeptides have been also characterized. One of the most exciting recent developments has been the discovery of a new type of long toxin from anemones belonging to the family Stichodactylidae. Several different laboratories have provided sequence data for these type 2 toxins, shown in Figure 2. They are characterized by: (1) lacking the first N-terminal amino acid, (2) possessing a lysyl residue between two half-cystines at position 4, (3) possessing a stretch of three acidic residues at positions 6–8, and (4) having a basic tetrapeptide sequence (Arg-Lys-Lys-Lys) at the C-terminus (Kem 1988*a*). The classification of long polypeptides into these two groups strictly follows current sea anemone systematics: type 1 polypeptides are found in the family Actiniidae and type 2 polypeptides occur in members of the family Stichodactylidae. Only about 30% homology exists between these two polypeptide types. Besides the six half-cystines, the other conserved equivalent amino acid residues are (using the numbering system for the *Anemonia* type 1 toxins): Asp 7, Asp or Glu 9, Gly 10, Pro 11, Arg 14, Ser or Thr 17, Gly 20, Gly 30, Trp 31, Ile or Val 41. Many of these residues (Gly and Pro particularly) are likely important for correct folding of the polypeptide chain, since they are almost always found in the hair-pin turns located on the molecular surface. Possible exceptions are the ionized residues and the tryptophan at position 31.

Schweitz et al. (1985) reported that the type 2 toxins isolated from *Heteractis "paumotensis"* are immunologically distinct from the type 1 toxins and also bind to a separate site on the sodium channel. The cDNA sequence (Noda et al. 1984) for the eel electric organ Na channel α-subunit revealed that this subunit contains four segments with very similar sequences. The Na channel is conceptualized as a pseudosymmetric tetrameric assembly. One inference that may be tentatively extracted from this model is that similar, but not necessarily identical, receptor sites for polypeptide toxins may occur in each segment or domain. This would provide an explanation for the probable occurrence of at least three polypeptide binding sites for: (1) sea anemone type 1 polypeptides, (2) scorpion β-toxins, and (3) sea anemone type 2 toxins and scorpion α-toxins. The relationships of *Goniopora* (a coral) and sea anemone short toxin binding sites to these three sites are not yet clear. The number of separate polypeptide toxin binding sites is still uncertain. Since the action of Hm-III is voltage dependent, it seems reasonable to assume that the sea anemone type 2 polypeptides must bind at a site other than the one occupied by the scorpion β-toxins. Clearly the anthozoan polypeptides acting upon different sites will be excellent molecular probes for investigating the topography of the sodium channel.

One interesting feature of the 5,000 dalton sea anemone polypeptides is the remarkable variation in crustacean and verte-

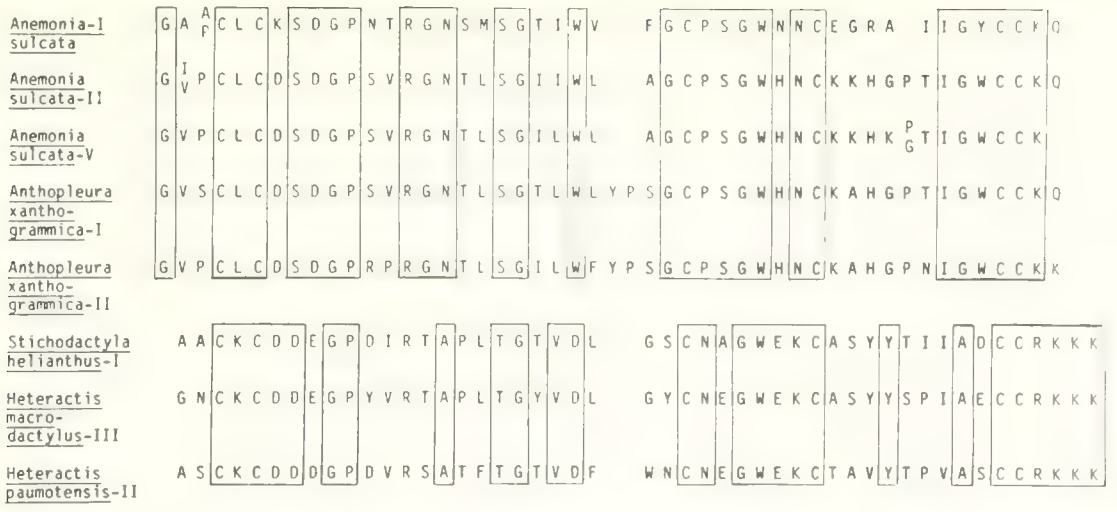
Anemonia-I sulcata
Anemonia sulcata-II
Anemonia sulcata-V
Anthopleura xantho-grammica-I
Anthopleura xantho-grammica-II
Stichodactyla helianthus-I
Heteractis macro-dactylus-III
Heteractis paumotensis-II
Abbreviations: A = alanine, C = half-cystine, D = aspartic acid, E = glutamic acid, F = phenylalanine, G = glycine, H = histidine, I = isoleucine, K = lysine, L = leucine, N = asparagine, P = proline, Q = asparagine, R = arginine, S = serine, T = threonine, V = valine, W = tryptophan, Y = tyrosine.

FIGURE 2. Amino acid sequences of sea anemone long polypeptide toxins affecting sodium channels. References: As-I, Wunderer and Eulitz 1978; As-II, Wunderer et al. 1976; As-V, Scheffler et al. 1982; Ax-I (anthopleurin A), Tanaka et al. 1977; Ax-II (anthopleurin B), Reimer et al. 1985; Sh-I, Kem et al. 1986*b*; Hm-III, Zykova et al. 1985; Hp-II, Wemmer et al. 1986.

brate toxicities between toxins (Table 2). This occurs regardless of whether the toxins are type 1 or 2. Comparison of the sequence of *Stichodactyla helianthus* I with *Heteractis "macrodactylus"* III reveals only 10 amino acid differences. Many of these seem trivial and unlikely to be responsible for the high crustacean, low mammalian activity of Sh-I or the opposite activity for Hm-III. The three major differences between these polypeptides are: (1) position 28 contains alanine in Sh-I but glutamic acid in Hm-III, (2) Sh-I possesses aspartic acid at position 11, whereas Hm-II has a tyrosine, and (3) Sh-I contains aliphatic hydroxyamino acids at positions 20 and 25, whereas Hm-III possesses the aromatic hydroxyamino acid tyrosine at these positions. Future structure-activity studies with these toxins might focus upon these particular structural differences and how they determine the toxicity spectrum of each toxin.

Several chemical modification investigations have been made on type 1 toxins in order to determine the importance of particular amino acid residues for receptor binding and activation. Unfortunately, the selectivity of modification and its influence upon secondary and tertiary structure have often not been fully analyzed. At present one can only conclude that adding bulky groups or neutralizing charge at Gly1 and Arg 14 significantly reduces toxicity. Esterification of both Asp 7 and Asp 9 of Ax-I abolishes its cardiac inotropic activity, but also disrupts the native structure of the toxin (Gruen and Norton 1985). It is now desirable to determine the effect of modifying Asp 7 or Asp 9 separately. Although the Asp 7–Arg 14 stretch has been implicated in receptor binding, this must be tested more critically in the light of a immunochemical study that found As-II, when bound to the sodium channel, is still readily accessible to rabbit antibodies specific for the Asp 9 and Glu 47 regions (El Ayeb et al. 1986).

In order to understand the different structural prerequisites for crustacean and vertebrate toxicity, we have embarked upon a program of generating mono-substituted toxin analogs, primarily by solid phase synthesis, for toxicity and sodium channel binding analyses. In this way, we hope to determine the importance of particular residues for toxin action and thereby iden-

TABLE II. Pharmacological properties of purified sea anemone long polypeptide toxins affecting sodium channels. LD_{50}'s are based on intrahaemocoelic (crab) or intraperitoneal injections; K_D = equilibrium dissociation constant; EC_{50} = median effective concentration increasing sodium fluxes. References: 1) Cg, Pf, Sh (Kem, submitted); 2) As, Ax, Sg (Schweitz et al., 1981); 3) Hm (Zykova et al., 1985); 4) Hp (Schweitz et al., 1985).

	LD_{50} (μg/Kg)		Rat Tissue Response (nM)	
Toxin	Crab	Mouse	Brain K_D	Heart EC_{50}
Type 1:				
Cg-II	0.2	>50,000	------	>1,000
Pf-I	0.4	>20,000	------	>1,000
As-I	2	4,000	7,000	-----
As-II	2	100	150	15
As-V	5	19	50	2
Ax-I	11	66	120	3
Ax-II	39	8	35	2
Type 2:				
Sh-I	0.3	>15,000	------	>8,000
Sg-I	7	> 2,000	> 10,000	-----
Hp-III	10	53	300	4,000
Hp-II	15	4,200	>100,000	5,000
Hp-I	36	145	900	3,000
Hp-IV	90	40	10,000	1,300
Hm-III	820	20	------	-----

As = Anemonia sulcata, Ax = Anthopleura xanthogrammica, Cg = Condylactis gigantea, Hm = Heteractis macrodactylus, Hp = Heteractis paumotensis, Pf = Phyllactis flosculifera, Sg = Stichodactyla giganteum, Sh = Stichodactyla helianthus.

tify the surface region of the toxin that interacts with the sodium channel. Our initial syntheses of native Sh-I showed it indistinguishable in toxicity, structure, and spectral characteristics from the natural toxin (Pennington et al. 1988).

Norton has pioneered in the NMR analysis of sea anemone polypeptide toxins. Gooley and Norton (1986) utilized two-dimensional proton NMR techniques to elucidate the secondary structure of Ax-I (anthopleurin A). Four different segments participate in a β-pleated sheet structure that had previously been detected but not localized by other spectroscopic methods. A hairpin turn was also located at positions 30–33, which separates two β-sheet strands. The Trp 23 and Trp 33 aromatic sidechains were shown to be close together by nuclear Overhauser enhancement measurements. Norton et al. (1986) used photochemically induced dynamic nuclear polarization NMR to show that both Trp residues are probably on the surface of the toxin, thereby generating a possible hydrophobic domain for binding to the Na channel. A similar surface is also present in the proposed binding domain of a scorpion Na channel toxin (Fonticella-Camps et al. 1981).

The secondary structures of type 2 long toxins are almost identical with that of the type 1 polypeptides (Nabiullin et al. 1982). Wemmer et al. (1986) recently investigated the secondary structure of *Heteractis* "*paumotensis*" toxin II by 2D-NMR methods similar to those previously employed by Gooley and Norton, and found essentially the same β-sheet structure for this type 2 toxin. They demonstrated the considerable analytical capability of the 2D-NMR method when they detected inconsistencies between their proton connectivity data relative to that expected from a published sequence (Schweitz et al. 1985). This led to an extensive revision of about half the polypeptide sequence.

The short sea anemone polypeptide toxins seem to affect Na channel inactivation in a manner similar to the long toxins. It is likely that short toxins bind to the same site as long type 1 toxins and scorpion α-toxins, since the latter toxins reduce their binding to nerve membranes. Their binding is also inhibited by membrane depolarization (Fujita et al. 1983). Although their amino acid sequences are very similar, only a three residue sequence at position 32–34 in Eq-I is found in the type 1 long toxins. X-ray structures are needed to determine if the receptor binding domains of long and short toxins have common features, in spite of their lack of sequence homology. The small size of these toxins makes them ideal for structure-activity studies. Possibly some variants of these small toxins will be found to possess mammalian inotropic activity.

The largest polypeptide neurotoxin so far isolated from a cnidarian is *Goniopora* toxin. Although this polypeptide also prolongs sodium action potentials by reducing the rate of inactivation, Gonoi et al. (1986) found that *Goniopora* toxin did not bind to the scorpion α-toxin (*Leiurus*) binding site. Since none of the sea anemone polypeptides were tested, it is not yet known if the *Goniopora* toxin binding site is unique. Further exploration of corals for toxins should be rewarding.

Clearly the cnidarians still represent a toxinological frontier, and it is anticipated that future investigations of other members of this phylum will reveal other toxins, likely polypeptide in nature, with interesting structures and modes of action. The anthozoan *Palythoa* has been shown to contain an extremely potent toxin that blocks the Na, K pump. Palytoxin is a complex, polyoxygenated non-peptide toxin, apparently synthesized by a bacterial symbiont. One then wonders what is present in the tentacles and nematocysts of this colonial organism. Although the soft corals and gorgonians possess a plethora of interesting terpenes, one wonders if the minute tentacles of these cnidarians might not also contain peptide chain toxins.

Nemertine Neurotoxins

Nemertines are active predatory worms that subdue their prey with a large, agile proboscis. Since nemertines lack other mechanical defenses, they also use this to protect themselves from other predators. The anoplan subphylum consists of paleonemertines (considered to have the most primitive body plan) and heteronemertines. These two classes of nemertines lack venom-injecting apparatus, so their integumentary toxins apparently serve for chemical defense. The enoplan subphylum consists almost entirely of hoplonemertines. Hoplos, the Greek word for "armed," is appropriately applied to these worms because their proboscis apparatus possesses one or more mineralized structures called stylets that pierce the prey's integument and thus facilitate envenomation. Practically all nemertines possess offensive and/or defensive toxins, except for some parasitic forms. Only a few species have been analyzed so far, due to difficulties in collecting and identifying these animals. Hoplonemertines use, both offensively and defensively, pyridine alkaloids some of which are potent nicotinic receptor agonists (Kem et al. 1976; Kem 1988*b*). Here I shall discuss primarily recent research on the heteronemertine polypeptide toxins from a nemertine (*Cerebratulus lacteus*) found along the east coast of North America.

The first *Cerebratulus* toxins to be isolated and characterized were the 6,000 dalton β-toxins that selectively paralyze and kill crustaceans, often at very low doses. Initial attempts at isolating these toxins from whole animal homogenates failed, but it was possible to obtain them from the integumentary mucous secretions. The three homologous variants are single polypeptides of about 55 residues crosslinked four times by cystinyl residues. Besides the eight half-cystines each toxin has a high proportion (~20%) of lysyl residues. The sequences of B-IV (the most abundant variant) and B-II (the most active variant) are known (Fig. 3). An interesting feature of both toxins is the presence of hydroxyproline at position 10; this was initially overlooked during the manual sequencing of B-IV without HPLC identification of the PTH-amino acids, but was established when B-II was sequenced on a Beckman 890 automatic sequencer using HPLC for product identification. It was recently shown by mass spectrometry that the 4-trans-hydroxyproline isomer is present at this position (Kem et al. 1986*a*). The possible functional importance of posttranslational proline hydroxylation in polypeptide toxins will be discussed below.

The secondary structures of these two β-toxins have been predicted and experimentally measured. The Chou-Fasman and other methods predicted about 30% helix and 30% β-sheet for these polypeptides, but it was found by both circular dichroism and laser Raman spectroscopy that β-sheet is absent and that 55–75% of the peptide bonds are hydrogen bonded in an α-helical manner (Kem et al., submitted). Since it is quite unusual for a polypeptide of this size to be largely α-helical, further NMR and crystallographic analyses of this toxin may be of general interest for understanding how predominantly α-helical proteins fold into their preferred tertiary structures.

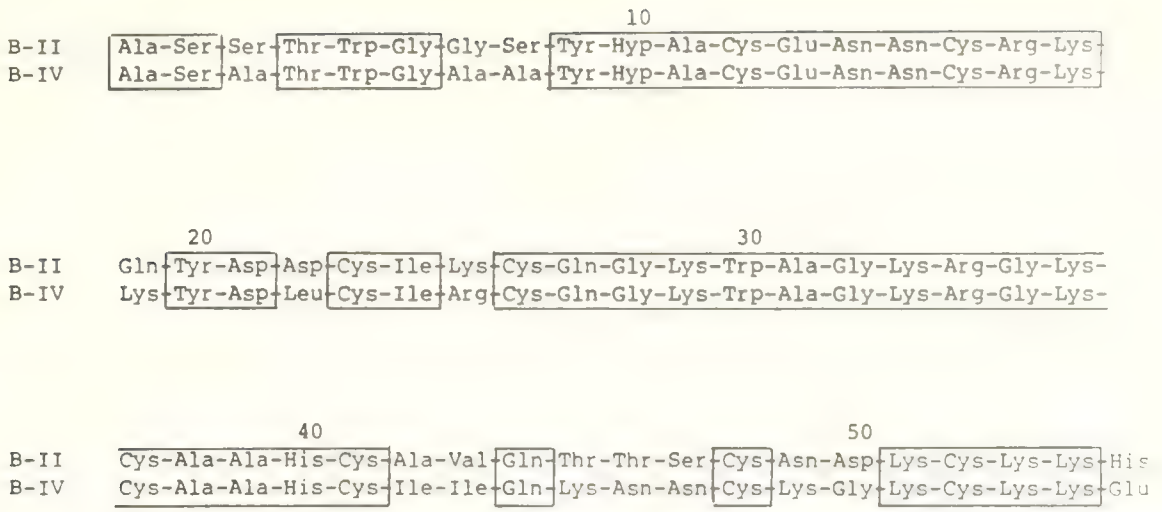

FIGURE 3. Amino acid sequences of the two major *Cerebratulus* (heteronemertine) neurotoxins (From Blumenthal et al. 1981). Hyp = hydroxyproline.

Analysis of the structural basis of toxicity began with chemical modification experiments, but these have provided only a limited insight due to difficulties in selectively modifying single residues of amino acids present as multiples. Nevertheless, it was possible to show that nitration of one (position 9) of the two tyrosyl side chains or alkylation of one (position 30) of the two tryptophanyl side chains destroys most of the activity of B-IV without affecting its secondary structure, as measured by circular dichroism (Blumenthal and Kem 1980). Blumenthal and Howell (1986) recently cloned a synthetic gene for *Cerebratulus* toxin B-IV in *E. coli* for the purpose of obtaining monosubstituted analogs by site-directed mutagenesis methods. Since this approach will allow manipulation of any desired position in the sequence, it should permit identification of the receptor binding domain on the surface of this toxin.

Progress has been recently made in elucidating the mechanisms of action of the heteronemertine neurotoxins. The predominant effects of the β-toxins in crustaceans are rapid development of hyperexcitability, tremors, and spastic paralysis, followed by flaccid paralysis and death. Nevertheless, when applied to isolated nerve bundles, the predominant effect is blockade of the action potential, sometimes preceded by repetitive spontaneous spiking. Toth and Blumenthal (1983) measured the specific binding of ^{125}I-B IV to lobster nerve vesicles and reported a K_D of 5–20 nM, which increased when extracellular potassium was elevated, presumably due to membrane depolarization. The density of saturable binding sites was similar to estimates of saxitoxin binding to the same preparation, which would be consistent with β-toxin binding to Na channels.

Another heteronemertine, *Lineus ruber* (Table 1), possesses a basic polypeptide toxin of about the same molecular size as that of *Cerebratulus*, but *Lineus* toxin delays the repolarization process during the action potential in a manner similar to anthozoan polypeptides. Characterization of this polypeptide has been largely hampered by difficulties in collecting satisfactory numbers of specimens for toxin isolation (Kem 1973).

Conus Neurotoxins

The gastropod genus *Conus*, comprising over 300 species, has a remarkable diversity and abundance of peptide and protein toxins. Cone snails are predators, envenomating their prey through a harpoon that is a modified radula (tooth). Most species feed on worms, others feed on other molluscs, and a few are able to paralyze and feed on fish. At this time the venom of only one species, *C. geographus*, has been systematically investigated. This cone is a piscivore with an amazing array of ion channel blocking peptides (Olivera et al. 1985). The major neurotoxic peptides of this snail affect various steps involved in neuromuscular transmission. The 27 residue ω-conotoxins block Ca channels at the nerve terminal, thereby depressing transmitter (ACh) release. The 13–15 residue α-conotoxins occupy the ACh binding site on the postsynaptic membrane, as do muscle relaxant drugs and the elapid snake α-toxins, thus preventing postsynaptic depolarization required for generation of the muscle action potential. As if this were not sufficient, *C. geographus* also injects its prey with a 22 residue μ-conotoxin that selectively blocks the muscle action potential! The covalent structures of representative α, μ, and ω-conotoxins ae shown in Figure 4.

Conus peptides are small and often present in minute (nanomole) quantities, so certain micromethods were particularly useful for their analysis. A multiplicity of peptides was separated by reversed phase HPLC. The dansyl method was useful for manually sequencing trace amounts of these peptides. Also, fast-atom bombardment mass spectrometry was used to determine the sequences of several peptides (Gray et al. 1981). Solid-phase peptide synthesis has permitted not only production of sufficient quantities for biological experiments, it has also been used to establish the disulfide pairings of the natural product (Nisiuchi et al. 1986).

General characteristics of these peptides are: (1) the carboxyl terminus is amidated, (2) there are many half-cystines, which are involved in disulfide crosslinking, (3) they are highly basic, and (4) the peptides frequently possess 4-hydroxyproline. With the exception of the C-terminal amidation, these characteristics also apply to the heteronemertine and some sea anemone polypeptides. Amidation, hydroxylation, and disulfide bonds are reasonable strategies for enhancing structural stability, but basicity is not so easily understood, as this also makes the peptides better substrates for trypsin-like proteases.

α-Conotoxin G1 (Gray et al., 1981)

1 5 10
Glu-Cys-Cys-Asn-Pro-Ala-Cys-Gly-Arg-His-Tyr-Ser-Cys·NH_2

Geographutoxin I (Sato et al., 1983)

1 5 10 15
Arg-Asp-Cys-Cys-Thr-Hyp-Hyp-Lys-Lys-Cys-Lys-Asp-Arg-Gln-Cys-
20
Lys-Hyp-Gln-Arg-Cys-Cys-Ala·NH_2

ω-Conotoxin GVIA (Olivera et al., 1984)

1 5 10 15
Cys-Lys-Ser-Hyp-Gly-Ser-Cys-Ser-Hyp-Thr-Ser-Tyr-Asn-Cys-Cys-
20 25
Arg-Ser-Cys-Asn-Hyp-Try-Thr-Lys-Arg-Cys-Tyr·NH_2

FIGURE 4. Amino acid sequences of the three major types of *Conus geographus* (gastropod) peptide neurotoxins blocking neuromuscular transmission.

The α-conotoxins have been most intensively studied. Using circular dichroism measurements, Hider estimated that roughly 50% of the 13 amino acid residues of conotoxin GI are in an α-helical structure, coinciding with the Chou-Fasman prediction that residues 5–11 would be helical. Gray et al. (1986) constructed a slightly different model for this peptide by attempting to maximize its similarity with a CPK model of the long loop of erabutoxin (a sea snake α-toxin), which is thought to possess much of the nicotinic receptor binding domain of this toxin. A double loop, tightly bridged by two disulfide bonds, was considered the most likely structure. In this model, the Arg 9 guanidinyl and the α-amino cationic groups are equivalent to the Arg 33 and Lys 47 cationic groups of erabutoxin. Synthetic replacement of Arg 9 in α-conotoxin with norleucine caused a 70% loss of activity; the remaining activity may be due to the ability of the Arg 2 guanidinyl group to substitute for Arg 9. It is also separated from the α-amino group by about 10 Å. The presence of two cationic groups separated by this distance is a common property of many nicotinic receptor drugs (Taylor 1985). Kobayashi et al. (1985) proposed two possible tertiary structures for this peptide, derived from 2D-NMR spectroscopy.

The μ-conotoxins selectively block muscle Na channels without affecting neuronal Na channels, except perhaps at much higher concentrations (Cruz et al. 1985; Ohizumi et al. 1986). This was the first pharmacological evidence that these two physiologically almost identical channels are not the same. The Kyoto laboratory of Noda et al. (1984) also showed that a cDNA clone of rat muscle Na channel RNA has a sequence different from that of rat brain. The μ-toxins bind to the same site as tetrodotoxin and saxitoxin (Ohizumi et al. 1986). Seven variants of this peptide were isolated and characterized by Cruz et al. (1985); by comparing their relative biological activities it can be concluded that the hydroxylation states of prolines 6 and 7 are not critical for activity. The importance of the remaining hydroxyproline at position 17 can not yet be evaluated, as all seven variants are hydroxylated at this position.

Besides the peptide toxins, cones also possess protein toxins. Striatoxin, isolated from *C. striatus,* is a 25,000 dalton glycoprotein that stimulates cardiac contractility. Hahin et al. (1981) studied the effects of two polypeptides affecting nerve action potentials in *C. striatus* venom. The smaller peptide which prolonged the duration of the neuronal action potential may be striatoxin. The larger polypeptide caused repetitive spiking. Complex affects of *C. striatus* venom on *Aplysia* neuronal soma potassium currents have also been reported (Chesnut et al. 1987). Phospholipase A activity is also present in this venom. Clearly *Conus* venoms, when investigated further, will yield many peptide chain toxins, in addition to the interesting peptides so far characterized.

Cephalopod Neurotoxins

For almost a century it has been known that octopuses paralyze their crustacean prey. Posterior salivary glands of the blue-ringed octopus (*Octopus maculosa*) possess tetrodotoxin, but the glands of all other cephalopods examined contain active peptide and protein toxins (Ghiretti 1960; Russell 1984). Octopus posterior salivary glands are large, the pair representing about 0.3% of the body weight. In comparison, the single posterior gland of the squid *Loligo pealei* is only about 0.03% of body weight.

Ghiretti (1959, 1960) was the first to attempt the isolation and characterization of these protein toxins, which he called "cephalotoxin." Using cuttlefish (*Sepia*) glands, the ammonium sulfate precipitable fraction was purified on a hydroxyapatite column. Starch gel electrophoresis of the active fraction revealed three components. In 1977, Cariello and Zanetti reported resolution of *O. vulgaris* venom into five components that were toxic to crabs. The two major proteins, designated α- and β-cephalotoxins, were acidic proteins with apparent molecular weights of 91,000 and 34,000 daltons, respectively.

The crab-paralyzing proteins of two other octopuses have also been partially purified. McDonald and Cottrell (1972) found that the crab paralytic activity from a North Sea octopus (*Eledone cirrosa*) eluted from a G75 Sephadex column as a single zone with an apparent molecular size of 30,000–70,000 daltons. This fraction was apparently unstable since they subsequently used a crude salivary gland homogenate to study venom effects upon a crustacean neuromuscular preparation. Muscle contractility to nerve stimulation was slowly but irreversibly blocked, even though the nerve retained its spontaneous activity. *Eledone* toxin could block either the glutamate receptor or its associated ion channel. Songdahl and Shapiro (1974) reported the purification of a 17,000 dalton polypeptide crab toxin from *O. dofleini,* a northwest Pacific species, using G50 Sephadex chromatography. Only one broad asymmetrical peak of absorbance was eluted, but the toxic fraction was reported to yield a single band during SDS gel electrophoresis. The isoelectric point of this component was 5.2 and the amino acid analysis after acid hydrolysis revealed large proportions of aspartic and glutamic acids.

Since Ghiretti (1959) found that glands of the cuttlefish *Sepia officinalis* possessed a similar protein, we investigated the sali-

vary gland toxicity of another decapod mollusc, the Atlantic squid *Loligo pealei*. A protein zone eluted from an Ultrogel Ac-44 column with an apparent molecular size of 30,000–50,000 daltons, as determined by its hemolytic activity profile. The major hemolytic component of this fraction possessed a pI of 7.8 (Kem and Scott 1980). At this time, it is uncertain whether this toxin is related to the previously described octopod toxins.

A major recurring problem with the cephalopod venom studies seems to be instability of the protein toxins. Although less convenient to obtain than homogenized glands, saliva secreted from live cephalopods would probably provide a much enriched toxin extract for purification. Since these glands also secrete digestive enzymes, either saliva or glandular homogenates should be purified initially in the presence of protease inhibitors. For *Loligo* gland homogenates, adding DFP and o-phenanthroline improves toxin stability (Kem, unpublished).

Eledoisin was apparently the first peptide to be isolated and characterized from a marine organism. This hypotensive undecapeptide has been found only in salivary glands of the octopus genus *Eledone* (Erspamer and Anastasi 1962).

Sea Urchin Neurotoxins

The spines and pedicellariae (pincer-like appendages between the spines) of various sea urchins have been implicated in envenomations, but the only species from which toxins have been isolated belong to the family Toxopneustidae. Two potentially injurious Indo-Pacific species (*Tripneustes gratilla* and *Toxopneustes pileolus*), and a Mediterranean species (*Sphaerechinus granularis*) have been investigated. The venomous globiferous pedicellariae were harvested either by blasting them free of the urchin with a forceful stream of sea water, or by painstakingly removing them by dissection. The pedicellariae were either homogenized *in toto* or allowed to autolyze in cold distilled water. A *Tripneustes* pedicellarial homogenate was resolved by G200 Sephadex chromatography into a high molecular weight (void volume) fraction possessing both kinin-releasing and hemolytic activities, and a smaller molecular size fraction lethal to mice (Feigen et al. 1970). Two groups have isolated a toxic protein from *Tripneustes gratilla*. Fleming and Howden (1974) isolated a 78,000 dalton (apparent molecular weight) protein by DEAE cellulose gradient chromatography; the isolated protein had an isoelectric point of 5.0. Mebs (1984) reported that the molecular size of the reduced and denatured lethal protein was 25,000 daltons by SDS–polyacrylamide gel electrophoresis and suggested that the native toxin may contain several 25,000 dalton subunits.

Nakagawa and Kimura (1982) partially purified a protein from the globiferous pedicellaria of *Toxopneustes pileolus* with an apparent molecular size (20,000 daltons) similar to Mebs's estimate for the *Tripneustes* toxin. Kimura et al. (1975) had previously separated the capillary permeability enhancement and smooth muscle contracting components from this urchin by G25 Sephadex chromatography.

A persistent complication with sea urchin toxin investigations has been their use of whole pedicellariae homogenates. To reduce the twin problems of contamination and proteolytic degradation, I devised another way to collect venomous constituents from a Caribbean sea urchin, *Lytechinus variegatus*. Animals are sequentially rotated in a small circular bowl containing 0.5 M ammonium acetate for several minutes, which causes the pedicellariae to hit nearby spines, triggering venom release. After using the same fluid for 15–20 urchins, the toxic proteins are recovered by dialysis and freeze-drying or by ammonium sulfate precipitation. The pedicellariae release venom only when simultaneously stimulated chemically and mechanically. Ammonium ions presumably depolarize a sensory receptor in the same manner as would potassium ions (Kem, in preparation).

At this point, none of the sea urchin protein toxins have been purified rigorously enough for chemical characterization. Nevertheless, it is clear that several different peptide chain toxins are present.

Annelid Neurotoxins

Small amounts of a very large (300,000 dalton apparent molecular size) protein have been partially purified from a European blood worm, *Glycera convoluta* (Bon et al. 1985). This neurotoxin could be a useful research tool, as it reversibly turns on transmitter release from nerve terminals. Due to the great difficulties anticipated in obtaining adequate quantities of this protein from its natural source, it would be an excellent candidate for application of the recombinant DNA strategy. Its pharmacological properties were recently summarized elsewhere (Kem 1988*b*).

CYTOLYSINS

Although neurotoxic polypeptides have attracted the most attention in the phylum Cnidaria, the variety of cytolytic proteins seems even greater. In most instances these substances are probably only secondarily lytic, their primary effects on prey and potential predator species being the production of pain, inflammation, and neuromuscular paralysis. Their lytic properties may largely function in the disruption of the predator or prey integument (including gills) and in the facilitation of digestion. In contrast to the neurotoxins, the protein cytolysins likely act without first binding to specific protein receptors on the target cell membrane. This conclusion is supported by studies of toxin action upon artificial lipid bilayers lacking proteins. Various experiments have also provided evidence that some sea anemone cytolysins preferentially bind specific membrane lipids, which may serve as membrane acceptors for these toxins (Linder et al. 1977).

The most extensively investigated cytolysin is a 17,000 dalton basic polypeptide isolated from the sea anemone *Stichodactyla helianthus*. Bernheimer and Avigad (1976) isolated this toxin by isoelectric focusing and reported its high affinity for sphingomyelin-containing membranes. Two laboratories subsequently demonstrated that the toxin forms a relatively non-specific ion channel in artificial lipid bilayers. Since the membrane conductance increase was found to be proportional to the toxin concentration raised to the third or fifth power, it was tentatively concluded that the ion channel is an aggregate of several monomers (Michaels et al. 1979; Varanda and Finkelstein 1980). *Stichodactyla helianthus* cytolysin was subsequently shown to consist of four different variants (Kem and Dunn, in press). Sh C-III accounts for about 80% of the total cytolysin present. Variant C-IV differed from the others possessing an additional 2,000 dalton chain length, some of which is accounted for by an N-terminal extension. This variant may be an incompletely

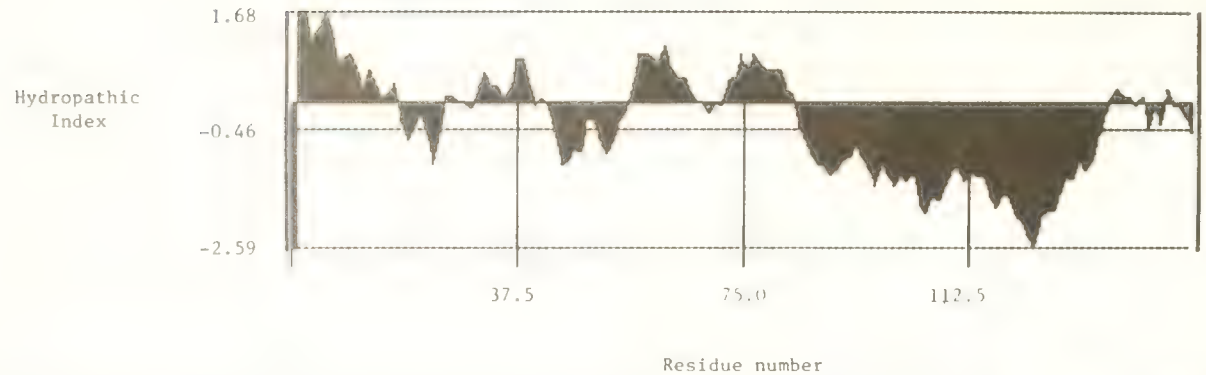

FIGURE 5 Hydrophobic and hydrophilic regions of *Stichodactyla helianthus* cytolysin III. The protein was analyzed using the SOAP program of Kyte and Doolittle. The hydropathic index is plotted as a moving mean of a 13 residue stretch of sequence. Hydrophobic regions have positive HI and hydrophilic regions have negative HI values (from Blumenthal and Kem 1983).

processed form of C-III, as the available sequence after the N-terminal extension is the same as for C-III. Sequencing of Sh C-III was a challenge not only due to its size (153 amino acid residues), but also because several hydrophobic peptides were difficult to isolate in reasonable yields for sequencing. A unique feature of this toxin is its lack of disulfide bonds. Another interesting aspect is the distribution of hydrophobic and hydrophilic amino acids (Fig. 5). At the N-terminus, there is a long nonpolar sequence predicted to be β-sheet. Subsequently, there are five shorter nonpolar segments that, as β-sheets, might be long enough to span the nonpolar region of a phospholipid bilayer. In contrast, the C-terminal third of this toxin is quite polar and is therefore expected to remain on the external surface of the target cell membrane. Secondary structure measurements on this protein indicated that 60–70% of the amino acids are hydrogen-bonded in a β-pleated sheet pattern. This is quite unusual for membrane-spanning proteins, having been observed only in one other group of channel-formers, the bacterial porins. Protein cytolysins apparently homologous with the *Stichodactyla* cytolysins have also been detected in several other sea anemone species (Bernheimer and Avigad 1981). It was recently proposed that this family of proteins be called "actinoporins" to indicate both their source (Order Actiniaria) and their major mode of action (Kem 1988*a*).

Bernheimer and Avigad (1976) partially purified a larger cytolytic protein (Table 1) from the acontiate sea anemone *Metridium*, and presented experimental evidence that this protein preferentially binds cholesterol. This is not without precedent, since microbial (polyene antibiotic) and plant (saponin) toxins also specifically interact with 3-β-hydroxysterols.

One of the most intricate and elegantly studied cytolytic venoms is that of a small Caribbean sea anemone *Aiptasia pallida* (see Hessinger et al. 1973; Hessinger and Lenhoff 1976). In this case, membrane disruption and subsequent cell death is produced by the synergistic interaction of at least three distinct proteins. A 43,000 dalton Ca^{++}-dependent phospholipase A_2 releases lysolecithin and fatty acids in the presence of two other components: (1) a basic polypeptide that apparently disrupts normal phospholipid packing in the membrane, and (2) a large (135,000 dalton), acidic (pI 4.8) protein that avidly binds fatty acids released by the phospholipase (Grotondorst 1979). Since it is likely that phospholipase A_2 also occurs in other cnidarian venoms, it will be of considerable interest to elucidate its chemical structure for comparison with vertebrate phospholipase A_2s.

Hydrozoans and scyphozoans generally contain very large protein cytolysins; many of these may be ion channel formers. There is some controversy over whether the cytolysins are tissue constituents or are localized within nematocysts. Several have been partially purified, but no chemical information is yet available. *Physalia* toxin, isolated from nematocysts, has been reported to consist of three different (125,000, 53,000, and 34,000 daltons) glycopolypeptide subunits (Tamkun and Hessinger 1981). Several of the jellyfish protein toxins excite a variety of cell membranes without causing significant cell lysis. If they also act as ion channel-formers, the pores they produce must be relatively small, selecting for ions other than sodium and calcium, or else they function only at membrane potentials near those of the resting cell. Otherwise the electrolyte balance of the cell would be so upset that colloid osmotic lysis would inevitably result. Biochemical and biophysical studies of these proteins would be of great interest; unfortunately none of the jellyfish toxins have yet been purified to an extent satisfactory for structural analyses.

The heteronemertine *Cerebratulus lacteus* possesses several homologous 10,000 dalton basic polypeptides called "A" toxins that lyse erythrocytes and other cells (Kem and Blumenthal 1978). These skin toxins probably act in nature on the mouth and gill membranes of potential predators, as they are ichthyotoxic when externally applied (Kem, unpublished). As with actinoporins, there are hydrophobic segments (residues 1–16 and 63–95) that interact with and penetrate the membrane phospholipid bilayer. The hydrophobic C-terminal segment 63–95, predicted to be α-helical, is also long enough to span the lipid bilayer. Dumont and Blumenthal (1985) found that removal of the C-terminal nine residues decreased lytic activity by 75%, while further proteolytic shortening of the toxin to residues 1–75 totally inactivated the toxin. The N-terminal segment also seems to span the membrane, since the Arg 13–Ser 14 peptide bond is cleaved by trypsin trapped within liposomes (Blumenthal 1982). Segments 1–16 and 63–95 were recently synthesized by solid-phase methods; segment 63–95 possessed only about 0.1% of native toxin hemolytic activity (Balasubramaniam et al. 1986).

The most recently discovered peptide cytolysins are the ichthyotixic pardaxins in the skin of a Mediterranean fish (Lazarovici et al. 1986; Thompson et al. 1986). These are short (33 residues) basic peptides that resemble the bee venom detergent peptide melittin.

DISCUSSION

Discovery of Marine Toxins

Although most of the toxins discussed in this article have rather rapid and conspicuous effects (such as convulsions, paralysis, and death), this could be partly due to the overreliance of toxinologists on bioassays particularly sensitive for neurotoxins acting on such peripheral receptors as sodium channels and nicotinic receptor-mediated channels. In the future, the most rewarding approach for discovering novel pharmacologically-active substances will be the use of new bioassays for detecting other receptor-mediated events, including those involving other neurotransmitters, modulators (including prostaglandins, leukotrienes, and platelet activating factor), and intracellular messengers. For instance, several useful snake toxins were discovered using assays sensitive to substances affecting acetylcholinesterase (Karlsson et al. 1984) and central nicotinic receptors (Ravdin and Berg 1979). *Cerebratulus* cytolysin and several other basic peptides were found to be potent *in vitro* inhibitors of the calcium-activated phospholipid-dependent protein kinase, an important intracellular regulator of cell function (Kuo et al. 1983). Obviously, the selection of receptor binding and biological assay preparations is of critical importance for discovery of new substances.

Another approach that should be productive is the screening of extracts from other species in a group already known to be toxic. In the case of sea anemones and cone shells, only a minority of toxin variants have high vertebrate toxicity; this suggests that isolation of isotoxins from diverse species may in some cases lead to the discovery of variants with high mammalian activity.

Venom constituents causing inflammation, pain, and cardiovascular shock have not yet been systematically investigated. Hartman et al. (1980) detected bradykinin immunoreactivity in a jellyfish venom; however, it is still uncertain whether the pharmacologically active protein constituents are structurally similar to kinins. Sublytic concentrations of *Physalia* venom stimulate histamine release from rat mast cells; further experiments will be required to determine if a factor other than the purified hemolysin is involved (Cormier 1984). Nakagawa (1985) showed that a 20,000 dalton protein toxin in sea urchin pedicellarial extracts was a potent histamine releaser; Feigen et al. (1970) previously had shown that similar extracts from another sea urchin species contain a potent kinin-liberating activity. Inflammatory substances, if they selectively affect a specific process, might become useful chemical tools for analyzing the mechanisms of inflammatory mediator release.

Perhaps one of the best approaches for finding new marine toxins is a zoological orientation: becoming familiar with literature on prey–predator interactions of marine organisms, their life-histories, and the anatomical structures associated with elaboration and release of venoms. Several comprehensive reviews and bibliographies providing such information have appeared in recent years (Hashimoto 1979; Russell 1984; Russell et al. 1984).

Peptide Chain Toxins as Chemical Tools

Exogenous proteins such as animal toxins seem unlikely candidates for becoming drugs, since their size makes them antigenic and also endows them with mediocre pharmacokinetic properties under most circumstances. It is as molecular probes of physiological and other processes that most of these substances will be useful. Snake α-bungarotoxin is a very useful polypeptide; its ability to bind irreversibly to skeletal muscle nicotinic receptors allows counting the receptors with iodinated toxin; in this way, muscles of myasthenia gravis patients were shown to be deficient in nicotinic receptors.

The anthozoan (sea anemone and coral) polypeptide toxins have become important ligands for investigating the topography of the sodium channel, since they apparently interact with at least two separate sites on this membrane protein. Although each of the four homologous domains of the sodium channel might possess binding sites for the various sodium channel toxins, this seems unlikely because the sequences of the channel domains vary considerably. Since all of the sea anemone polypeptides seem to affect inactivation in a similar manner, one wonders if the inactivation process involves a concerted movement of these different domains. Fluorescence energy-transfer and affinity-labelling approaches with anthozoan and scorpion polypeptides should allow measurements of distances between the binding sites and identification of the domain possessing a binding site for a particular toxin.

Since the μ-conotoxins selectively block skeletal muscle sodium channels, these peptides will be particularly useful in the experimental analysis of neuromuscular transmission processes under more normal conditions than has been previously possible. In the past, muscle action potentials were eliminated by reducing transmitter release (lowered Ca^{++} and elevated Mg^{++}), or by reducing the postsynaptic response with a nondepolarizing muscle relaxant. Muscle contractions resulting from action potentials were also suppressed by disrupting the transverse tubule-sarcoplasmic reticulum system with glycerol, which often depolarized the resting potential. Normal endplate responses can now be observed in the presence of synthetic μ-conotoxin.

The ω-conotoxins, because they block neuronal calcium channels which are resistant to nifedipine and other similar agents, will be very useful chemical tools for investigating release of transmitter substances from nerve terminals. Now that these otherwise scarce peptides can be synthesized in relatively large amounts by solid phase methods, they should stimulate investigations of a variety of calcium channels, some of which are implicated in cardiovascular and other disease states.

Phylogenetic Spectrum of Toxicity

One reason tetrodotoxin and batrachotoxin are so useful for investigating sodium channels is that they are generally active on sodium channels, regardless of target organism or tissue. This seems to be much less the case for the peptide chain toxins. Presumably their interactions with binding sites involve the simultaneous generation of many more intermolecular contacts than for smaller molecules, so some small steric differences between receptor sites might have deleterious consequences for polypeptide binding affinity.

The best documented phylogenetic difference in toxicity is that of the sea anemone polypeptide neurotoxins (Table 2). Among both types of sea anemone long polypeptides, there are tremendous differences in crustacean and vertebrate toxicity. These often seem inversely related. Another example of an extreme phylogenetic spectrum of toxicity occurs with *Conus* venoms (Endean and Rudkin 1965). Only venoms of fish-eating

cones are particularly toxic for vertebrates. The piscivores represent only perhaps 10% (about 30) of the total number of described *Conus* species. The remaining cones (a nearly virgin group for future investigations), which prey upon worms or molluscs, seem to have venoms specialized for the paralysis of their preferred prey. It will be of considerable interest to determine if these latter species have fundamentally different peptide chain toxins, or if the toxins are just homologs of the piscivorous *Conus* peptides that possess higher affinities for receptors in worms and molluscs. Although sea anemone long polypeptides with exceptional crustacean or vertebrate toxicity are clearly homologous, they nevertheless show remarkable phylogenetic specificity in many instances. Again, vertebrate toxicity is more the exception than the rule. Screening the polypeptide toxins from a variety of nemertine species also might turn up variants with significant toxicity towards vertebrates or arthropods.

Peptide Chain Toxins as Models for Analysis of Secondary and Tertiary Structure

Many toxins I have considered possess relatively high proportions of certain secondary structures; conformational analysis of these molecules may provide new insights regarding the importance of particular molecular properties for predicting secondary and tertiary structures from amino acid sequences. In particular, the high proportion of α-helix in *Cerebratulus* β-toxins requires explanation. Investigations of *Stichodactyla* cytolysin, a membrane-spanning polypeptide with a high proportion of β-sheet, could provide considerable insight into the mechanism of membrane penetration by protein segments that are not α-helices. Finally, the functional importance of hydroxyproline residues in determining secondary and tertiary structure and upon peptide stability can also be readily investigated in the sea anemone, nemertine, and *Conus* peptide chain neurotoxins.

Possible Therapeutic Applications of Peptide and Protein Toxins

There are problems inherent in the use of exogenous peptides or proteins as therapeutic agents. Whether administered acutely or chronically (for weeks or months), a major anticipated problem is low bioavailability, particularly when administered orally. Even when stable to proteolytic enzymes in the gastrointestinal tract, the peptide or protein must be absorbed into the systemic circulation to reach its intended site of action. Chronic administration of a peptide drug would likely induce an immune response, terminating its use. The chemical synthesis of peptides may also be relatively expensive.

Recently, the ability to produce large quantities of hormonal and other biomedically important proteins by recombinant DNA technology has made the testing and possible therapeutic use of peptides and proteins much more feasible. This, in turn, has stimulated considerable research on the associated problem of increasing the bioavailability of such drugs by increasing the rate and extent of systemic absorption and by reducing their destruction by tissue and plasma proteases. The oral bioavailability of insulin, interferons, and other proteins has been significantly increased by co-administration with lipoidal adjuvants (Muranishi 1985) or by encapsulation within lipsomes or crosslinked polymers (Saffran et al. 1986). Also, peptides and proteins have been found to enter the systemic circulation rapidly when administered to the nasal mucosa as emulsions (Moses et al. 1983). Peptides and proteins can be made less susceptible to proteases by blocking the terminal amino and carboxyl groups and by introducing less susceptible amino acids at critical points. In this way captopril, with its prolyl-containing peptide bond, was designed as an effective inhibitor of angiotensin-converting enzyme (Cushman et al. 1978). When a therapeutic peptide is chemically synthesized, D-amino or N-methyl amino acids can be used to replace protease susceptible L-amino acids (Veber and Freidinger 1985).

Protein Engineering

The remarkable confluence of several new technologies—particularly (1) protein and DNA sequence methods, (2) 2D-NMR and x-ray tertiary structural techniques, (3) computer graphics, (4) cDNA cloning and expression methods, and (5) peptide synthesis—has resulted in a new level of capability in manipulating peptide-protein structures, both experimentally and theoretically. The term "protein engineering" is a designation for this approach, which is being intensively developed within pharmaceutical research institutes. In most cases the focus has been on design of new inhibitors of enzyme active (or allosteric) sites, and has involved the molecular graphics analysis of x-ray crystallographic images of these sites (Hol 1986). However, protein engineering approaches also are applicable to peptide chain toxins that act on membrane receptor proteins. The most essential prerequisite for this approach is a high resolution tertiary structure. This serves as a conceptual guide for structure-activity analysis of the ligand surface, using chemical synthesis or site-directed mutagenesis replacement of specific surface residues.

Another potential contribution of the molecular graphics approach is in the prediction of antigenic sites. In general, polar highly mobile segments, including hairpin turns, frequently are major components of an antigenic site, although debate continues about the validity of this generalization (Novotny and Haber 1986; Van Regenmortel 1986).

Therapeutic Potential of Sea Anemone Polypeptides for Treatment of Congestive Heart Failure

For some time, pharmaceutical and other laboratories have sought agents to replace the relatively toxic digitalis glycosides. These drugs block the sodium pump, thereby leading to a transient elevation in the concentration of intracellular sodium ion during the cardiac action potential. This probably stimulates a sarcolemmal membrane Na_i for Ca_o exchanger that increases $(Ca)_i$, thereby stimulating the force of ventricular contraction. Unfortunately, the digitalis glycosides also cause cardiac arrhythmias due to an indirect effect of blocking the sodium pump—namely, depolarization of the resting membrane potential, which generates ectopic pacemakers and tachyarrhythmias.

The sea anemone polypeptides As-II and Ax-I enhance myocardial contractility in a novel fashion. By delaying inactivation of the myocardial sodium channel, they transiently enhance sodium influx during the action potential, which probably elevates $(Ca)_i$ by stimulating the same Na_i-Ca_o exchanger (Shibata and Norton 1982). These polypeptides have a significantly better margin of safety than do the digitalis glycosides. Nevertheless, by prolonging the duration of the action potential, they also cause extrasystolic contractions and, at higher concentrations,

myocardial fibrillation (Hashimoto et al. 1980). Ironically, these toxic effects may occur because the polypeptides are too effective in delaying the closing of the sodium channels. A major challenge in designing less toxic compounds resembling these polypeptides will be to minimize toxicity, perhaps by finding analogs with less ability to inhibit the sodium inactivation process. It has been shown in frog nerve that As-II treatment produces at least two modified sodium channel populations (Ulbricht and Schmidtmayer 1981). The major population simply shows a slower inactivation rate than before polypeptide treatment, while a smaller (but more damaging) population of channels never inactivates during the course of a prolonged nerve action potential. This non-inactivating population probably causes the arrhythmias. Further basic research using patch clamp methods is needed to determine if this permanently open sodium channel state can be avoided by manipulating the polypeptide's structure. It is conceivable that lower efficacy analogs might have less toxicity.

The application of protein engineering to sea anemone polypeptides seems possible in the very near future, since the high resolution x-ray (or 2D-NMR) tertiary structure of one of these two inotropic polypeptides should soon be available. This will certainly facilitate structure-activity studies to identify the receptor binding domain on the polypeptide's surface. Once this domain is localized, efforts can be made to remove other portions of the molecule in order to reduce its antigenicity and improve its bioavailability. Some synthetic analogs may be found with decreased cardiotoxicity relative to inotropic activity. Finally, the neuronal potency of the polypeptide analogs should also be assessed since neurotoxicity may often be associated with inotropic agents that affect sodium channels.

The Design of Tumor Specific Cytotoxins: A Speculation

Cytotoxins such as ricin and diptheria toxin would become useful chemotherapeutic agents if they could be made to attack tumor cells selectively. In order to accomplish this, many laboratories are developing molecular conjugates (such as immunotoxins), in which the toxin is attached to a molecule specific for the tumor cell surface. This approach has considerable potential because a single molecule of ricin or diptheria toxin is capable of killing a cell by inhibiting its protein synthesis. Certain membrane-active cytotoxins are also extremely potent. For instance it can be calculated that a single ion channel formed by *Stichodactyla* cytolysin should be sufficient to kill a cell. Consequently, it seems at least plausible that successful conjugation of such an ion channel complex with a selective ligand for a tumor cell could be an equally effective chemotherapeutic agent. One advantage of utilizing such a channel-former is that it would need only to penetrate the cell membrane rather than to be translocated across the membrane into the cytoplasmic compartment.

CONCLUDING COMMENTS

Only a few peptide chain toxins occurring in marine organisms have been investigated in any detail at this time. Many more await discovery, a process that will be facilitated by the introduction of new screening bioassays capable of detecting more subtle effects on organisms (and their constituent cells and tissues) than is possible with currently used bioassays. Now that several types of peptide chain toxins have been isolated and their amino acid sequences have been elucidated, it is possible to proceed to the investigation of their tertiary structures and the identification of their receptor-binding domains. The synthesis of peptide chain toxin analogs by both chemical and recombinant DNA approaches should provide important leads for designing new drugs and pesticides with unique modes of action.

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The Phylogenetic and Biomedical Significance of Extended Neuropeptide Families

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INTRODUCTION

Neuropeptides are synthesized by neurons as segments of larger precursor proteins. Even as they are translated from mRNA, these precursors enter the secretory pathway to be packaged in vesicles within which they are enzymatically processed to smaller, biologically active, molecules. The vesicles are transported to the axonal terminals whence the peptide products are secreted, either into the circulation (i.e., as neurohormones), or adjacent to their target cells (i.e., as neurotransmitters) (Gainer et al. 1985). In either case, a chain of events leading to an effect is initiated in the target tissue when peptides bind to receptors that may vary from one target site to another. Thus, the characteristically diverse functions of neuropeptides may be allocated to different tissues and independently regulated.

Peptides seem to occur in families characterized by similarities of sequence, and this essay is a consideration of that tendency. The structural affinities are examined for their regularity and possible genetic basis. The taxonomic range of peptide families is considered, and we ask, in particular, whether they transcend phyla. The biomedical significance of invertebrate peptide families is presented as a special case of such transcendence; that is, a peptide family is biomedically significant when it occurs and is active in mammals. This proposition is illustrated in terms of the molluscan family of peptides related to FMRFamide.

THE ELEMENTARY PEPTIDE FAMILY

Definition

The smallest definable peptide family unit is a set of peptides occurring in a single species. The family members in that species are recognized by their substantial similarities of sequence. No other structural generalities seem to hold. That is, the members of an elementary peptide family can be relatively long or short; their length can be uniform or variable; and their characteristic common residues can be at the C-terminal, the N-terminal, or distributed along the length of the peptide (e.g., Fig. 1; Fig. 2).

Genetic Basis

Neuropeptide families arise from the operation of two phenomena that lead to changes in the sequences of peptide products: gene duplication and subsequent nucleotide substitutions. The extent of the gene duplication that has occurred varies from one peptide family to another, as is illustrated by the following examples.

At one extreme, the duplication of the gene can be complete, involving the entire genomic organization of exons (coding regions) and introns (non-coding regions). The gene family in *Aplysia californica*, encoding homologous polyprotein precursors that produce egg-laying hormone in the bag cells and related peptides in the atrial gland, is a classic example of a peptide family arising by complete duplication of the gene (Scheller et al. 1983; Mahon et al. 1985; Nagle et al. 1986; Rothman et al. 1986). Even when the precursors of related peptides have relatively low sequence homology—as in the case of pancreatic polypeptide (Leiter et al. 1985) and neuropeptide Y (NPY) (Larhammer et al. 1987)—a close similarity in the organization of the exons encoding the peptides is suggestive of complete gene duplication. Complete gene duplication seems also to be responsible for the emergence of the family of neurohypophyseal hormones (Acher 1984).

Duplications within genes also occur. At least part of the tachykinin family in mammals seems to have been generated in this way; i.e., substance P and substance K occur in a common precursor, but they are encoded on different, presumably duplicated, exons (Nawa et al. 1984; Krause et al. 1987). Relatively short segments within exons may also be duplicated: e.g., the MSH sequence in the gene encoding pro-opiomelanocortin (POMC) (Nakanishi et al. 1979); the enkephalin sequences in proenkephalin A and B (Noda et al. 1982; Horikawa et al. 1983); sections of the small bag cell peptides in the ELH precursor (Scheller et al. 1983), and perhaps the multiple copies of caerulein in two of its precursors (Richter et al. 1986). An outstanding illustration of this process is the FMRFamide precursor from *Aplysia californica* which contains 28 copies of the tetrapeptide and its processing signals (Taussig and Scheller 1986).

The genetic relationship between the members of a peptide family is not invariably obvious. For example, while preproenkephalin A and B each contain a set of three or more enkephalin-containing (EC-, or opioid) peptides, the most striking similarity between them is the structural organization of the genes encoding them. Moreover, both of these precursors are quite different from pro-opiomelanocortin (POMC), which produces only a single copy of the enkephalin sequence. Against great odds, Numa (1984) makes a convincing case for homology.

Also obscure is the relationship between the precursors of gastrin and cholecystokinin (CCK). In the pig, for instance, both precursors contain, near their C-terminals, the dodecapeptide: -Gly-Tyr-Met-Asp-Phe-*Gly-Arg-Arg*-Ser-Ala-Glu-Glu (where the italicized residues are signals for cleavage and amidation; Yoo et al. 1982; Gubler et al. 1984). This common sequence aside, the two precursors are extraordinarily dissimilar, even within the N-terminal portions of the hormones (Fig. 2). Yet the probability is very small that the long sequences in common are attributable to convergence. Since the C-terminal pentapeptide of gastrin/CCK and its attached processing signal -Gly-Arg-Arg- appear in caerulein (sequence in Fig. 6), which is secreted by some amphibian skins (Erspamer et al. 1984), this sequence may be a long-conserved message that has been shuffled frequently among various unrelated genes (Doolittle 1987).

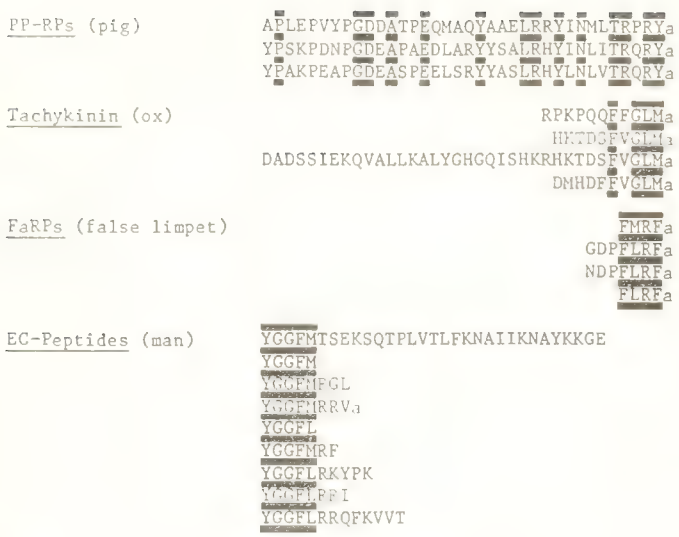

FIGURE 1. A sampler of elementary peptide families; the characteristic common sequence of each is shown. Note that the relative lengths of the peptides within a family, the uniformity of their lengths, and the location of the common sequences are *not* general features of elementary peptide families. References: pancreatic polypeptide-related peptides (PP-RPs) of pig (in Greenberg et al. 1987); tachykinins of ox (in Nakanishi 1987; except the long analog—neuropeptide K—which is a projection from the pig: Tatemoto et al. 1985); enkephalin containing (EC-) peptides of man (Numa 1984); FMRFamide-related peptides (FaRPs) of the false limpet *Siphonaria pectinata*, a pulmonate snail (Price et al. 1987*a*). Amino acids are represented by their one-letter symbols.

ALLOCATION OF FUNCTION

The members of an intraspecific neuropeptide family are characteristically produced in particular cells and act as receptors in certain tissues to elicit specific effects. Thus, the duplication of a neuropeptide gene, and the independent evolution of the resulting sequences, can lead efficiently to new agents with new actions on different tissues. This is, *a priori*, the biological justification for the occurrence of peptide families.

Even when the DNA replication is intragenic, histological and functional specificity are provided by alternative processing of both the mRNA and the protein precursor. For instance, three unique preprotachykinins are expressed by a single gene in the rat (Krause et al. 1987; see also Nawa et al. 1983; Nakanishi 1987); and the differential processing of the precursor proenkephalin A in different tissues is well described (reviewed by Udenfriend and Kilpatrick 1984).

In summary, no elementary neuropeptide family can be said to have been completely described until the genes encoding it and the products that are finally produced are known. In only a very few cases have the requisite data been obtained with material from a single species: the EC-peptides of rat and man; the egg-laying hormone-like peptides of *Aplysia californica*; and the FMRFamide-related peptides of the same gastropod.

THE EXTENDED NEUROPEPTIDE FAMILY

Homologs of the peptides discovered in one species are routinely found in other, more or less related, species. [In vertebrates, the succession frequently proceeds from the ox (a bountiful and convenient source of material) to the rat (the primary experimental model).] The set of all such homologs in all species is the extended peptide family, and its taxonomic limits should be at least those of the phylum.

Ideally, generalizations about extended peptide families should be based upon comprehensive comparative data. However, the available sample is deficient in two respects. First, the complete elementary family of very few neuropeptides is known in any species, a matter discussed above. Second, the selection of species examined tends to be patchy simply because a phylogenetically comprehensive sample is rarely an aim in neuropeptide biochemistry. Thus, the group of Vittorio Erspamer is interested in natural products and sees the amphibian skin "... as an enormous storehouse of biogenic amines and polypeptides" (Erspamer et al. 1984). Through the years, they have tested the skins of well over a hundred amphibians, and have found seven tachykinins (in addition to the caerulein- and bombesin-like peptides and others) (Erspamer 1981; Erspamer et al. 1984). But the tachykinins of amphibian brain have never been sequenced. In contrast, the mammalian tachykinins were particularly sought in the rat brain and were sequenced much later in the ox (reviewed by Erspamer 1981; Buck and Burcher 1986).

Nevertheless, a few extended peptide families have been sufficiently studied that three generalizations can be made, as follows.

1. The number of species-dependent sequence differences varies independently from one member of a peptide family to another. For example, although human and rat NPY are 100%

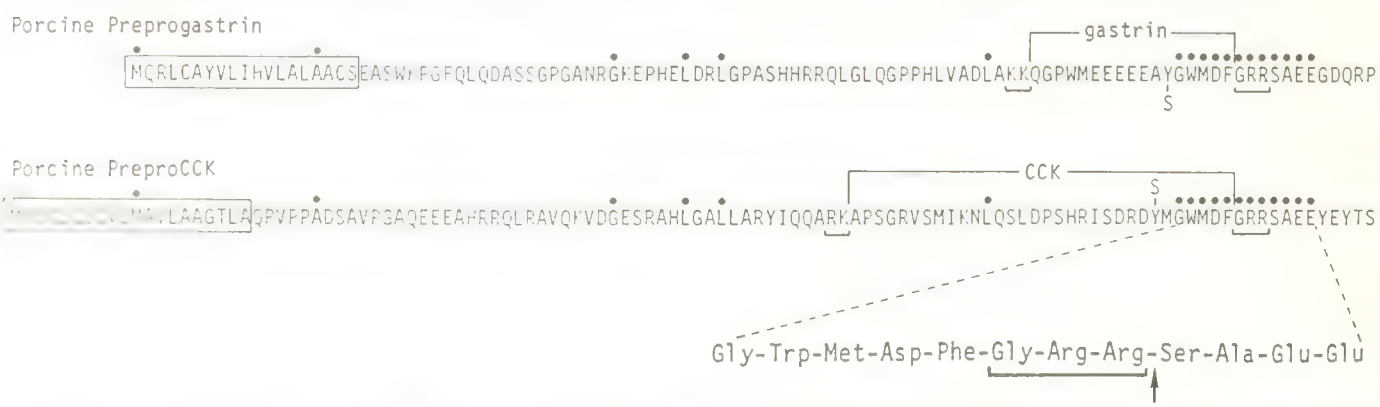

FIGURE 2. The precursors of gastrin (preprogastrin) and of cholecystokinin (preproCCK) in the pig. Residues common to both precursors are indicated with a large dot, the common C-terminal dodecapeptide is set out in three-letter symbols and expanded. The processing signals for cleavage (paired basic amino acids) and amidation (glycyl residue) are underlined; the sulfated tyrosyl residues are indicated 'Y-S'; and the hormonal products are bracketed. The signal peptide is boxed. References: preprogastrin, Yoo et al. 1982; preproCCK, Gubler et al. 1984.

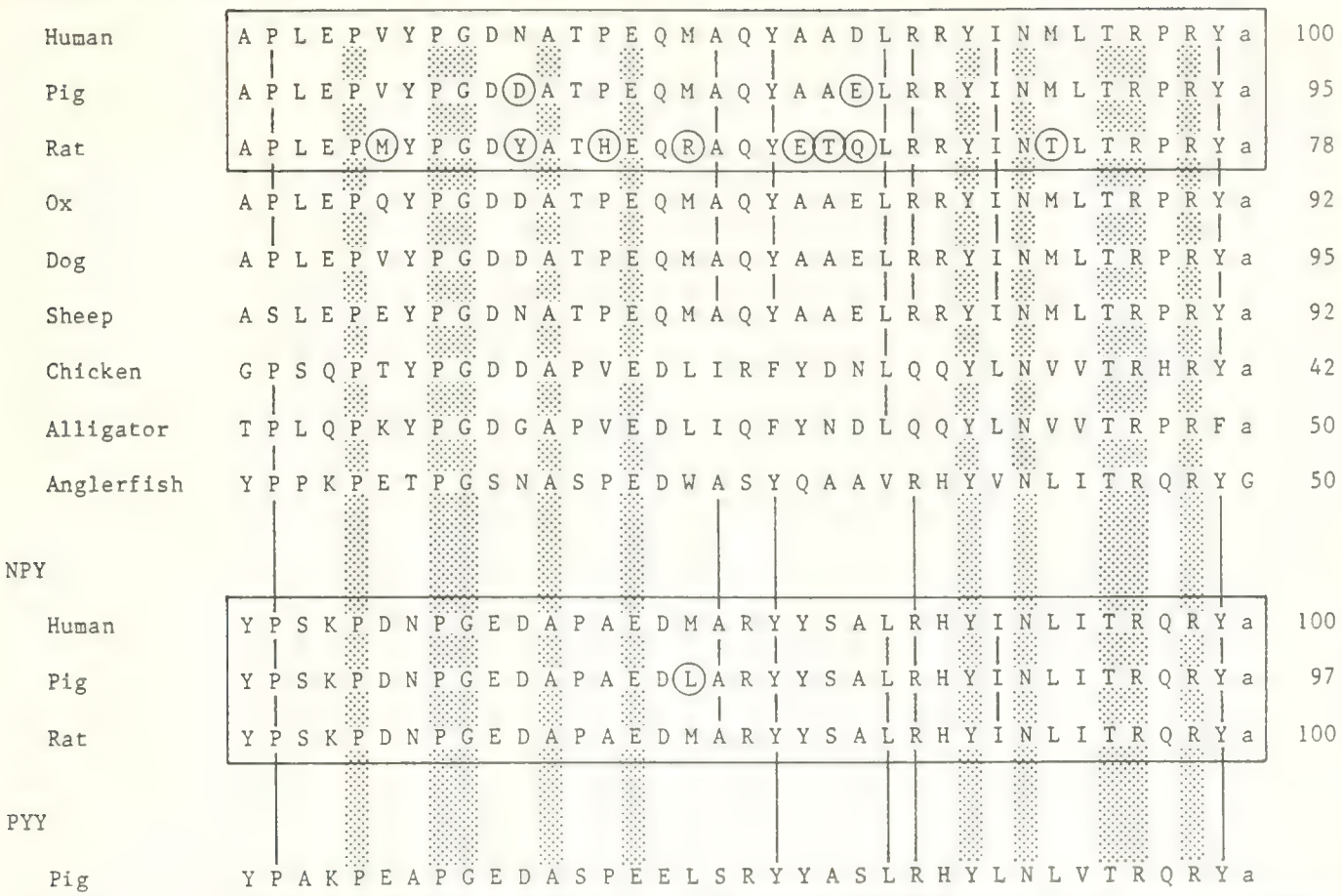
PANCREATIC POLYPEPTIDE
Human A P L E P V Y P G D N A T P E Q M A Q Y A A D L R R Y I N M L T R P R Y a 100
Pig A P L E P V Y P G D D A T P E Q M A Q Y A A E L R R Y I N M L T R P R Y a 95
Rat A P L E P M Y P G D Y A T H E Q R A Q Y E T Q L R R Y I N T L T R P R Y a 78
Ox A P L E P Q Y P G D D A T P E Q M A Q Y A A E L R R Y I N M L T R P R Y a 92
Dog A P L E P V Y P G D D A T P E Q M A Q Y A A E L R R Y I N M L T R P R Y a 95
Sheep A S L E P E Y P G D N A T P E Q M A Q Y A A E L R R Y I N M L T R P R Y a 92
Chicken G P S Q P T Y P G D D A P V E D L I R F Y D N L Q Q Y L N V V T R H R Y a 42
Alligator T P L Q P K Y P G D G A P V E D L I Q F Y N D L Q Q Y L N V V T R P R F a 50
Anglerfish Y P P K P E T P G S N A S P E D W A S Y Q A A V R H Y V N L I T R Q R Y G 50
NPY
Human Y P S K P D N P G E D A P A E D M A R Y Y S A L R H Y I N L I T R Q R Y a 100
Pig Y P S K P D N P G E D A P A E D L A R Y Y S A L R H Y I N L I T R Q R Y a 97
Rat Y P S K P D N P G E D A P A E D M A R Y Y S A L R H Y I N L I T R Q R Y a 100
PYY
Pig Y P A K P E A P G E D A S P E E L S R Y Y A S L R H Y L N L V T R Q R Y a

FIGURE 3. The pancreatic polypeptide-related peptides of vertebrates: a comparison of sequences. Shading indicates invariant residues; a line joins those residues with at least 69% similarity across the family. The column on the right shows the percentage of sequence similarity with the appropriate human peptide (i.e., pancreatic polypeptide or NPY = 100%). Compare, especially, the PPs and NPYs of human, pig, and rat; circled residues are different from human. References to most of the sequences are in Greenberg et al. 1987; for human NPY, see Minth et al. 1984; for rat NPY, see Larhammer et al. 1987. The residues are represented by their one-letter symbols.

homologous, human and rat pancreatic polypeptide are only about 78% homologous (Fig. 3). In contrast, the number of substitutions in the small family of neurohypophyseal hormones is relatively constant (Acher 1984).

2. Even when homologous peptides in an extended family have less than half their residues in common, their characteristic pattern is retained. Two easily recognizable elements of pattern are the overall length of the homologous peptides, and the distribution of the conserved residues within the sequence (e.g., Fig. 3 and Table 1; see also Acher 1984).

3. The functions of homologous peptides may change markedly from species to species, especially in divergent phyla. Indeed, the functions ascribed to the PCH/AKH-like hormones of arthropods (Table 1) are invariably the assayable effects used in their purification. But this is a conclusion that might well remain tentative, for the physiological roles of most neuropeptides are not well understood.

THE FMRFamide-RELATED PEPTIDES: AN EXTENDED FAMILY IN MOLLUSCS

The tetrapeptide Phe-Met-Arg-Phe-NH_2 (FMRFamide) was discovered about a decade ago in the ganglia of *Macrocallista nimbosa*, the sunray venus clam (Price and Greenberg 1977). From the first, FMRFamide was recognized as a potent and versatile agonist in molluscs. It has varied effects and mechanisms of action on molluscan hearts, visceral and somatic muscles (reviewed by Greenberg et al. 1983; recent references: Muneoka and Saitoh 1986; Smith and Hill 1987) and on molluscan neurons (reviewed by Walker 1986; for recent references: Colombaioni et al. 1985; Belardetti et al. 1987; Brezina et al. 1987*a*, *b*; Cottrell and Davies 1987). In a recent examination of the action of FMRFamide on pleural sensory neurons in *Aplysia*, the eicosanoid metabolites of arachidonic acid were shown to be a new class of second messengers (Piomelli et al. 1987).

The roles of FMRFamide in molluscan organismic physiology are still emerging. First, the peptide seems to regulate (and often to inhibit) those functions involved in feeding and digestion in gastropods, from the feeding motor program in the central nervous system (Cooke et al. 1985; Murphy et al. 1985; Lloyd et al. 1987), to peripheral structures, such as the buccal musculature, gut, or salivary glands (Austin et al. 1983; Lehman and Greenberg 1987; Bulloch et al. 1988). Occasional evidence has also implicated the FMRFamide-like peptides in reproduction (Lehman and Price 1987; Lehman and Greenberg 1987;

TABLE 1. PEPTIDES RELATED TO ADIPOKINETIC HORMONE (AKH) AND RED PIGMENT CONCENTRATING HORMONE (RPCH) IN ARTHROPODS.

Peptide[a,b]	Source (genus)	Amino acid sequence[c]
AKH	(Schistocerca/ Locusta)	pGlu - Leu - Asn - Phe - Thr - Pro - Asn - Trp - Gly - Thr - NH_2
AKH-II-L	(Locusta)	pGlu - Leu - Asn - Phe - Ser - Ala - Gly - Trp - NH_2
AKH-II-S	(Schistocerca)	pGlu - Leu - Asn - Phe - Ser - Thr - Gly - Trp - NH_2
AKH-M	(Manduca/ Heliothis)	pGlu - Leu - Thr - Phe - Thr - Ser - Ser - Trp - Gly - NH_2
HTF	(Nauphoeta/ Blaberus)	pGlu - Val - Asn - Phe - Ser - Pro - Gly - Trp - Gly - Thr - NH_2
HTF-II	(Carausius)	pGlu - Leu - Thr - Phe - Thr - Pro - Asn - Trp - Gly - Thr - NH_2
MII	(Periplaneta)	pGlu - Leu - Thr - Phe - Thr - Pro - Asn - Trp - NH_2
MI	(Periplaneta)	pGlu - Val - Asn - Phe - Ser - Pro - Asn - Trp - NH_2
RPCH	(Pandalus)	pGlu - Leu - Asn - Phe - Ser - Pro - Gly - Trp - NH_2

[a] HTF: hypertrehalosaemic factor; M: myoactive factor.
[b] References to most of the identifications are in Schooneveld et al. 1987; for HTF, see Gäde and Rinehart 1986; for HTF-II see Gäde and Rinehart 1987.
[c] Solid box, invariant residues; dashed box, ≥66% similarity.

A. B. Brussaard, personal communication) and, in gastropods, the process of emerging from and withdrawing into the shell (Lehman and Greenberg 1987). Finally, FMRFamide is, classically, a cardioactive peptide, and evidence that it actually functions in molluscs as a cardiovascular or branchial regulator is growing (Weiss et al. 1984; Furukawa and Kobayashi 1987; Krajniak and Bourne 1987; other references in Smith and Hill 1987, and Smith 1987).

Six FMRFamide-related peptides (FaRPs) are now well-established as occurring in molluscs (Greenberg et al. 1987). They include a pair of tetrapeptides (FMRFamide and FLRFamide) and a quartet of heptapeptides (XDPFLRFamide, where X can be a glycyl (G), seryl (S), asparaginyl (N), or pyroglutamyl (pQ) residue). We have been assiduous about determining the distribution of these FaRPs among the Mollusca (Price 1986; Eberink et al. 1987; Price et al. 1987*a*, *b*) and have found that the higher taxa fall into three clear groups based on the particular analogs that are present and on their relative tissue concentrations (Table 2).

TABLE 2. DISTRIBUTION OF FMRFAMIDE-RELATED PEPTIDES (FARPS) AMONG THE MOLLUSCA.

Group	FaRP	Relative amount
Pulmonate gastropods	FMRFamide	100
	FLRFamide	15
	X-DPFLRFamide[a]	100–300[b]
Opisthobranch gastropods	FMRFamide	100
	FLRFamide	< 5
Other molluscs[c]	FMRFamide	100
	FLRFamide	15

[a] X can be Gly, Ser, Asn, or pGlu. Most pulmonates contain two heptapeptides, but one and three are also possible, and no species contains both the glycyl and pyroglutamyl residues. The amount of each heptapeptide is roughly that of FMRFamide.
[b] The following taxa were sampled: Polyplacophora; Bivalvia; Cephalopoda; Prosobranchia (Gastropoda).

In essence, the heptapeptides occur only in the subclass Pulmonata (including the veronicellids and onchidellids) in amounts approximating those of FMRFamide; and the ratio of FLRFamide to FMRFamide is three to five times smaller in the Opisthobranchiata than in any other major taxon.

Certain unusual features of the gene encoding the FMRFamide precursor in *Aplysia californica* (see Taussig and Scheller 1986) have led us to speculate (Price et al. 1987*b*) about the genetic basis of the systematic distribution of FaRPs shown above. First, the *Aplysia* precursor contains only one copy of FLRFamide, but 28 copies of FMRFamide (Taussig and Scheller 1986). Thus, if all of the copies were processed, no more than 4% of the activity could be due to FLRFamide [the exact amount measured would depend on the assay used (Greenberg et al. 1987)] and it could easily be missed (e.g., Lehman et al. 1984).

A second characteristic of the known FMRFamide precursor—a long stretch of the gene that includes about 19 copies of FMRFamide that are highly repetitious (Taussig and Scheller 1986)—we attribute to a relatively recent gene duplication. If the iterations are deleted, the resulting "ancestral" precursor would contain only nine copies of FMRFamide to one of FLRFamide (details in Price et al. 1987*b*), and this is the ratio observed in most molluscs (Table 2).

Finally, the single copy of FLRFamide occurs at the 5′ end of the FMRFamide precursor, just downstream from a copy of a peptide that ends: -Gly-Tyr-Leu-Arg-Phe-NH_2 (Taussig and Scheller 1986). Since the pulmonate heptapeptides are extended analogs of FLRFamide, we have suggested that they arose through the duplication and subsequent modification of the 5′

terminal of the ancestral gene. These speculations about the origin of the extended family of FaRPs in molluscs remain to be tested.

TRANSPHYLETIC NEUROPEPTIDE FAMILIES

Although the details are far from complete, the essential story remains that virtually all of the species in a phylum will contain the same group of extended peptide families, and that the extremes of variation within each family will be sufficiently narrow that any member peptide will be readily recognized as such. We must next ask whether, and how frequently, peptide families extend into other phyla, and whether we can differentiate between transphyletic extensions that are homologous and those that occur by chance.

The wide and persistent application of immunochemical techniques has produced a large body of evidence suggesting that neuropeptide families are virtually ubiquitous (e.g., Greenberg and Price 1983). But identifications by immunochemistry are not nearly stringent enough to sustain that notion. Therefore, the cases discussed below are those in which the transphyletic members of peptide families have actually been sequenced.

Tachykinins

These peptides are recognized by the general similarity of their structures, by their common C-terminal sequence: -Phe-X-Gly-Leu-Met-NH_2 (Fig. 4), and by their pharmacological actions: e.g., lowering of blood pressure, contraction of smooth muscle, and stimulation of salivation (Erspamer 1981; Buck and Burcher 1986).

All of the known tachykinins occur in vertebrates, except (curiously) for the first tachykinin to have been sequenced; i.e., eledoisin, a product of the posterior salivary glands of *Eledone moschata,* an octopus (Erspamer 1949; Erspamer and Anastasi 1962). Eledoisin is not only limited in its occurrence to molluscs, it seems further restricted within that phylum to a particular gland in a single species. For example, no peptide was detected in several other tissues in *Eledone,* nor in the posterior salivary glands of other species of cephalopods, nor in the hypobranchial glands of *Murex* (Erspamer 1949, 1981). This singular distribution of eledoisin has, however, never been adequately tested; for as Erspamer (1981) noted, the nervous system in these animals was not examined, and detection was limited to mammalian bioassay systems. From another perspective, the absence of eledoisin from the posterior salivary glands of even closely related species exemplifies the idiosyncratic distribution of peptides in epithelial glands (e.g., atrial gland peptides [Nambu and Scheller 1986]; peptides of amphibian skin [Erspamer et al. 1984]).

Kream et al. (1986), using newer assay techniques, found very little substance P-like immunoreactivity in the pedal ganglia of *Mytilus edulis,* and none of it was authentic substance P. However, the antiserum used in this study was only 0.01% cross-reactive with eledoisin and physalaemin, so the search may have been too narrow. In conclusion, the probability that only one structurally unexceptional tachykinin would occur in molluscs, and then by chance, is very small. A further search for invertebrate tachykinins seems warranted.

EC-Peptides

Material reactive with antisera to the enkephalins or endorphins has been detected in most of the major invertebrate phyla

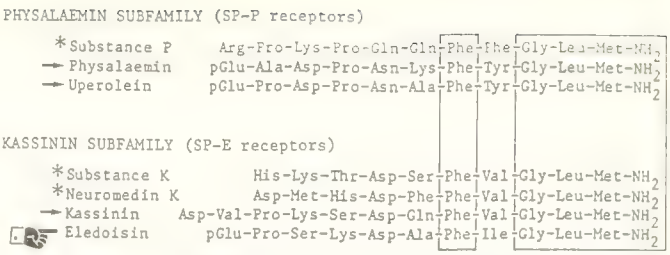

FIGURE 4. The tachykinin peptide family: from mammalian brain (*), amphibian skin (arrow), and the posterior salivary glands of *Eledone moschata* (Cephalopoda, Octopoda) (index finger). The solid boxes enclose invariant residues characteristic of this peptide family. References to the identification of the peptides are in Erspamer 1981; for the amphibian peptides see, especially, Erspamer et al. 1984. The subfamilies and receptor types are described by Buck and Burcher (1986).

(reviewed by Greenberg and Price 1983; and Greenberg et al. 1986). In molluscs, these preliminary identifications have been pursued. First, several biochemical and pharmacological studies have suggested that enkephalin-like peptides act as modulators of dopaminergic systems in molluscs (reviewed by Leung and Stefano 1986). Later, Met-enkephalin, Leu-enkephalin and Met-enkephalin-Arg^6-Phe^7 were purified and identified (by HPLC elution time, receptor binding activity, and sequence) in the pedal ganglion of *Mytilus edulis.* Moreover, the levels of the peptides were equivalent to those in mammalian brain (Leung and Stefano 1983, 1984).

This identity of mammalian and molluscan peptides should be considered in the context of enkephalin variation within the vertebrates. Levels of Leu-enkephalin are relatively low in amphibian brain (*Bufo*; Kilpatrick et al. 1983) and, in fact, the gene encoding proenkephalin in *Xenopus laevis* contains an extra copy of Met-enkephalin in place of Leu-enkephalin, which is lacking (Martens and Herbert 1984). But the usual concentrations of Leu-enkephalin occur in reptiles (Lindberg and White 1986). Thus, Leu-enkephalin in vertebrates (exclusive of that derived from prodynorphin) might either have appeared first in reptiles, or have been selectively lost in amphibians. In any event, these findings do not preclude the separate appearance of Leu-enkephalin in molluscs. Moreover, the similarities between amphibian, reptilian, and mammalian proenkephalins are very much more striking than the differences. In summary, the EC-peptides may well occur widely in invertebrates.

Anthropodan FaRPs

Immunoreactive FMRFamide has been detected in various crustaceans and insects over the years (references in Greenberg et al. 1985; Van Deijnen et al. 1985; Kobierski et al. 1987; and Marder et al. 1987), and FMRFamide has been shown to modulate the motor pattern of the stomatogastric ganglion of a crab (Hooper and Marder 1984). In the last two years, however, several arthropodan FaRPs have finally been sequenced (Fig. 5).

One decapeptide, isolated from the heads of cockroaches (*Leucophaea maderae*), was detected and assayed by its inhibition of spontaneous rhythmicity of the hindgut of this insect (Holman et al. 1986); it is therefore called leucomyosuppression (LMS). Very recently, Nambu et al. (1987) identified a nonapeptide with FMRFamide-like immunoreactivity in extracts of

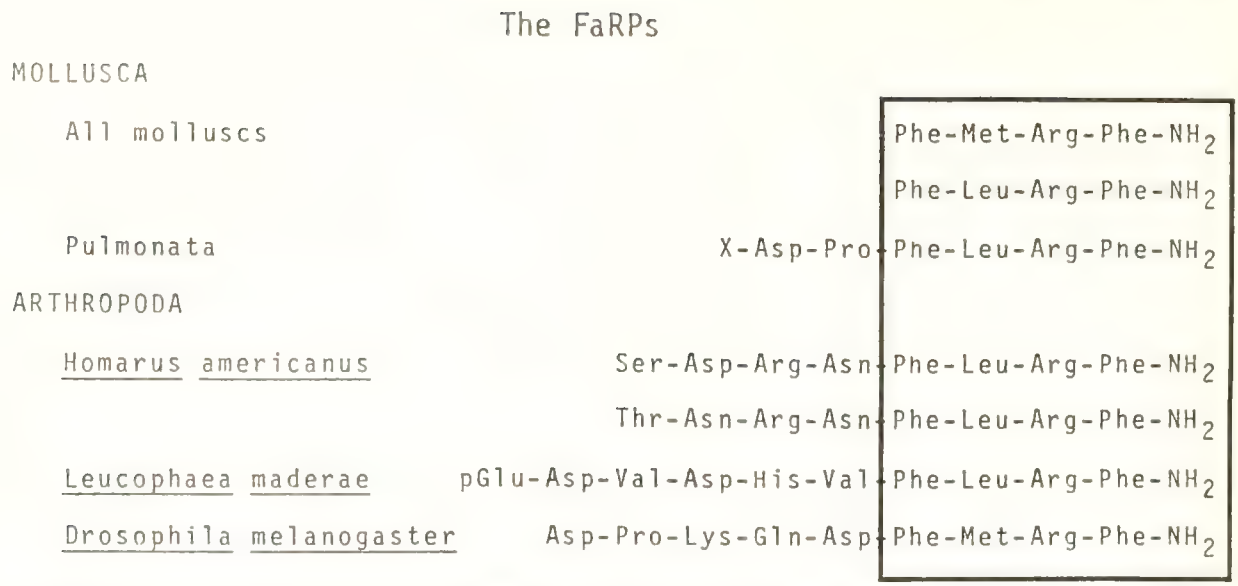

FIGURE 5. The transphyletic family of FMRFamide-related peptides (FaRPs). The C-terminal tetrapeptide characteristic of the family is boxed. X can be a glycyl, pyroglutamyl, seryl, or asparagyl residue. Molluscan sources of FaRPs are listed in Price et al. 1987*b*; for the lobster peptides, see Trimmer et al. 1987; for the cockroach, Holman et al. 1986; and for the fruitfly, Nambu et al. 1987.

the fruitfly *Drosophila melanogaster*. The gene has also been isolated and sequenced. It is reminiscent of the FMRFamide gene from *Aplysia*, encoding a precursor containing about 12 copies of some seven different analogs of FMRFamide (Schneider and Taghert 1988). Finally, two octapeptides were identified in the pericardial glands of the American lobster (*Homarus americanus*), again by their reactivity with antibodies to FMRFamide (Trimmer et al. 1987). All of these peptides are N-terminally extended analogs of FLRFamide or FMRFamide and, in that sense, are similar to the heptapeptides found in the molluscan pulmonates (Fig. 5). At this writing none of these arthropod peptides has been tested for their cross-reactivity in molluscan bioassays.

The similarity of the arthropodan and molluscan FaRPs, and their occurrence in closely related protostomous phyla, suggests that the assemblage is part of an authentic transphyletic peptide family.

Antho-RFamide and the L5 Peptide

The immunoreactive FMRFamide observed in several species of coelenterates (Grimmelikhuijzen 1985; Grimmelikhuijzen and Graff 1985), was finally identified, in the sea anemone *Anthopleura elegantissima* and the sea pansy *Renilla kollikeri*, as the tetrapeptide pGlu-Gly-Arg-Phe-NH_2 (pQGRFamide) (Grimmelikhuijzen and Graff 1986; Grimmelikhuijzen and Groeger 1987). Notwithstanding the C-terminal dipeptide, considerations of genetics, bioactivity and immunoreactivity indicate that pQGRFamide has little relationship to the FMRFamide-related peptides (Greenberg et al. 1987). Coincidental to the discovery of the coelenterate peptide, neuron L5 in the left upper quadrant of the abdominal ganglion of *Aplysia californica* was shown to stain strongly with an antiserum to FMRFamide (Brown et al. 1985). Although the mRNA expressed in L5 does not encode FMRFamide, it does contain one copy of a peptide that terminates -Gln-Gly-Arg-Phe-NH_2 (Shyamala et al. 1986). This terminal sequence—which would account for the cell's FMRFamide-like staining—is also the same as that in antho-RFamide since N-terminal pyroglutamic acid (pGlu) residues form by the cyclization of glutamine (Gln).
the same as that in antho-RFamide since N-terminal pyroglutamic acid (pGlu) residues form by the cyclization of glutamine (Gln).

The wonder that such similar tetrapeptide sequences would appear in such disparate phyla is dampened by the high probability that it could occur by chance (Price 1983). Moreover, the number of species in which either of the peptides has been seen could hardly be smaller; and there is as yet no evidence, from coelenterates or molluscs, of a *family* of antho-RFamide-like peptides.

The Leucosulfakinins (LSKs)

Two sulfated neuropeptides (LSK and LSK-II) with homology to gastrin and CCK were recently identified in the brain and corpora cardiaca of a cockroach, *Leucophaea maderae*. The effect of the peptides is to increase the frequency and amplitude of the spontaneous contractions of the hindgut of the cockroach (Nachman et al. 1986*a*, *b*).

The sequence homology between the leucosulfakinins, gastrin, CCK, and caerulein is substantial. Moreover, the potency of these cockroach peptides is (like the binding of the vertebrate peptides to receptors in ectothermic animals; Vigna et al. 1986), dependent on the sulfation of the tyrosyl residue which is six positions from the C-terminal (Nachman et al. 1986*a*, *b*). However, the LSKs differ critically from gastrin and CCK in that the C-terminal sequence is -Met-*Arg*-Phe-NH_2, rather than -Met-*Asp*-Phe-NH_2 (Fig. 6). From this difference, we draw three conclusions:

First, lacking the acidic aspartyl group, the LSKs cannot have the biological activity of gastrin or CCK (Morley 1968). As a corollary, the LSK receptors in the cockroach hindgut should

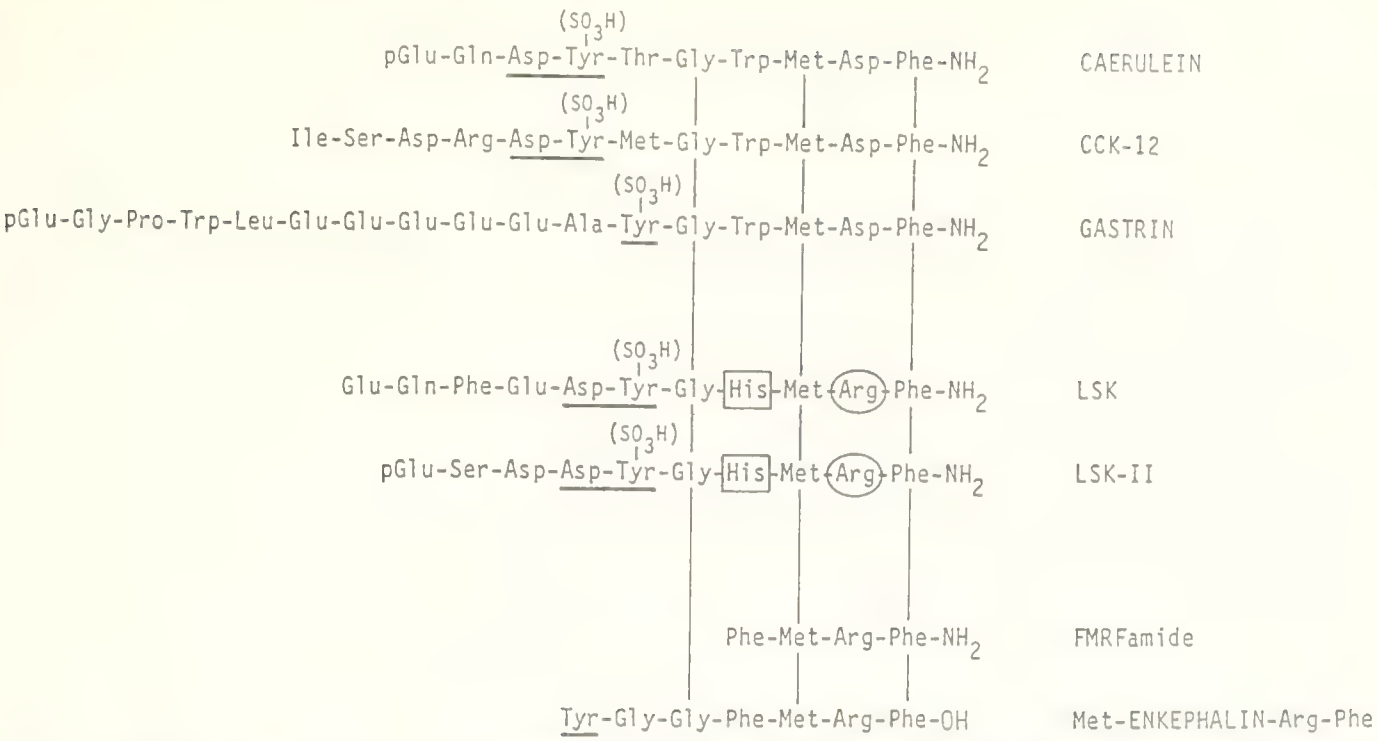

FIGURE 6. Similarities in sequence between the leucosulfakinins (LSK; LSK II), the gastrin/CCK/caerulein family, the FMRFamide-related peptides, and the EC-peptides. Identical residues are connected; among them are pGlu and Gln, which are both encoded as Gln, the pGlu forming through cyclization of Gln. The sulfated tyrosyl residues and related amino acids are underlined; note the alignment of these residues. The Arg and His residues in the C-terminal tetrapeptides of LSK and LSK-II are, respectively, circled and boxed to emphasize critical differences between these peptides and the gastrin/CCK and FMRFamide families.

be insensitive to CCK, gastrin, and caerulein—and they are (Nachman et al. 1986*a*, *b*).

Second, the LSKs will also be unreactive with C-terminally directed antisera to gastrin or CCK, and cannot, therefore, be responsible for most of the immunoreactive gastrin and CCK reported so frequently in insects (references in Nachman et al. 1986*b*).

Third, the C-terminal tripeptide of the leucosulfakinins is the same as that of FMRFamide (Fig. 6) in which the basic arginyl residue is essential for all biological activity in molluscs. However, the substitution of histidine at the Phe[1] position of FMRFamide causes the leucosulfakinins to be weakly active (about 0.15%) in bioassays for FMRFamide (Greenberg et al. 1987).

In summary, the leucosulfakinins have structural affinities with both the FaRPs and the gastrin/CCK-like peptides, but functional affinities with neither. Moreover, the leucosulfakinins have the same relationship (and irrelationship) with the EC-peptide Met-enkephalin-Arg[6]-Phe[7] (Fig. 6).

Structural similarities between functionally diverse peptide families have often been noted. For example, FMRFamide has been weakly linked with gastrin/CCK (Price and Greenberg 1977; Dockray and Dimaline 1985), the EC-peptides (Doble and Greenberg 1982), SCP_B (Morris et al. 1982), the pancreatic polypeptide-like peptides, and others (Greenberg et al. 1987). We conclude that, no matter whether certain fragments of sequence have been strongly conserved or have arisen independently in different phyla, their frequent recurrence reflects fundamental structural requirements for association between peptides and proteins. As a corollary, protein receptors with complementary requirements should appear as frequently.

BIOMEDICAL SIGNIFICANCE OF INVERTEBRATE PEPTIDE FAMILIES

The discovery of a transphyletic neuropeptide is almost always an event of interest to students of comparative biology or evolution. But the special case in which an invertebrate peptide family is found to extend to mammals, or to have substantial pharmacological actions on mammalian systems, is likely to be of biomedical importance, as well. At present, the molluscan FaRPs are the only invertebrate peptide family with sequenced analogs in vertebrates. These analogs are described below.

LPLRFamide, A Chicken Brain Peptide

Immunoreactive FMRFamide has been detected in the nervous tissues of every major class of the subphylum Vertebrata (Table 3). Often this immunoreactivity has been associated with known vertebrate peptides, particularly γ_1MSH and the pancreatic polypeptide-related peptides (reviewed by Greenberg et al. 1987). Finally, in 1983, Dockray et al. identified the pentapeptide Leu-Pro-Leu-Arg-Phe-NH_2 (LPLRFamide) in chicken brain, and it is therefore the first vertebrate peptide to have been detected and assayed by its reactivity with an antiserum to an invertebrate neuropeptide. Several new peptides, immunochemically related to FMRFamide and LPLRFamide, but otherwise uncharacterized, have subsequently been detected in the avian central nervous system (Dockray et al. 1986).

TABLE 3. IMMUNOREACTIVE FMRFAMIDE IN THE VERTEBRATES.

Class	No. species	bio	icc	RIA	chr	seq	References
Osteichthyes	2	+	+				1, 7
Amphibia	1		+	+			3,4
Aves	1		+	+	+	a	2, 3, 4
Mammalia	7	+	+	+	+	b	1, 3, 4, 5, 6, 8, 9

bio: bioassay; icc: immunocytochemistry; RIA: radioimmunoassay; chr: chromatographic separation; seq: sequence.
a: LPLRFamide Leu-Pro-Leu-Arg-Phe-NH_2
b: A18Fa Ala-Gly-Glu-Gly-Leu-Ser-Ser-Pro-Phe-Trp-Ser-Leu-Ala-Ala-Pro-Gln-Arg-Phe-NH_2
F8Fa Phe-Leu-Phe-Gln-Pro-Gln-Arg-Phe-NH_2
References: 1: Boer et al. 1980; 2: Dockray et al. 1983; 3: Dockray et al. 1981*a*; 4: Dockray et al. 1981*b*; 5: Lundberg et al. 1984; 6: O'Donahue et al. 1984; 7: Stell et al. 1984; 8: Triepel and Grimmelikhuijzen 1984; 9: Yang et al. 1985.

Since the sequences of LPLRFamide and FMRFamide are so alike, the qualitative similarity of their effects on both molluscan and vertebrate systems was to have been expected. Nevertheless, although the two peptides are roughly equipotent in their effects on mammalian neurons and blood pressure (Dockray et al. 1983; Barnard and Dockray 1984), the presence of a prolyl residue in the Phe^1 position reduces the potency of LPLRFamide on molluscan bioassays to less than 1% of that of FMRFamide (Greenberg et al. 1987). Of course, the physiological role of LPLRFamide in *chickens* has never been investigated and remains entirely unknown.

TABLE 4. ACTIONS OF FMRFAMIDE IN VERTEBRATES.

System observed	Effect*	Reference
Central nervous system		
Intracerebroventricular, intracisternal, or intrathecal injection (mice and rats)		
Morphine- and stress-induced analgesia	−	Tang et al. 1984; Kavaliers and Hirst 1986
Opiate-, deprivation-, and stress-induced feeding and locomotor activity	−	Kavaliers and Hirst 1985; Kavaliers et al. 1985; Kavaliers and Hirst 1986
Grooming-related activities	+	Raffa et al. 1986
Drinking (blocked by saralasin)	+	M. I. Phillips (personal communication)
Blood pressure	+	Barnard and Dockray 1984; Wong et al. 1985
Growth hormone secretion	+	Ottlecz and Telegdy 1987
Pharmacology and electrophysiology		
Opioid receptor binding (rabbit)	−	Zhu and Raffa 1986
Medullary neurones (rat)	+	Gayton 1982
Nucleus tractus solitarius neurons (rat)	−	Dockray et al. 1983
Retinal ganglion cells (goldfish)	+	Walker and Stell 1986; Stell et al. 1984
Peripheral circulation		
Mean arterial pressure and heart rate (rat)	+, [0]	Mues et al. 1982; Barnard and Dockray 1984; [Sander and Giles 1985]
Vasodilation of superfused vascular beds (rat)	+	Koo et al. 1983
Peripheral hormonal effects		
Glucose-stimulated insulin and somatostatin release (perfused rat pancreas)	−	Sorenson et al. 1984

* (+) induction, excitation, or increase; (−) inhibition, depression, or decrease; ([0], [ref.]) no effect in the dog.

FMRFamide-related Nociceptive Peptides of Mammals

The similarity in sequence between FMRFamide and the EC-peptide Met-enkephalin-Arg^6-Phe^7 (YGGFMRF) led to the notion that the two peptide families and their receptors had coevolved from an ancestral peptide and its receptor (Greenberg et al. 1981). Although this was an attractive hypothesis, there was plenty of evidence that the apparent homology was an example of convergence (reviewed by Greenberg et al. 1986). Recent investigations of both molluscan and mammalian systems have restored interest in the connection between the opioid peptides and the FaRPs (reviewed by Greenberg et al. 1987). Two sets of complementary mammalian experiments were especially compelling and fruitful.

First, in a series of behavioral studies with mice, intracerebroventricularly administered FMRFamide reduced the analgesia, feeding and locomotor activity induced by morphine, opioid peptides, stress or deprivation (Kavaliers and Hirst 1985, 1986; Kavaliers et al. 1985).

Second, in rats, centrally administered FMRFamide reduced the analgesia induced by Met-enkephalin-Arg^6-Phe^7 and morphine. Moreover, an immunoreactive FMRFamide-like material, extracted from ox brain and partially purified, could also attenuate an opioid-induced analgesia in rats. Centrally administered FMRFamide antiserum induced its own long-lasting analgesia and decreased the tolerance to morphine analgesia, thus suggesting that the rat brain also contains endogenous, FMRFamide-like, antinociceptive factors. Indeed, perfusion with morphine caused the release of immunoreactive FMRFamide (Tang et al. 1984).

In the denouement, two peptides, identified by their reactivity with an antiserum to FMRFamide, were isolated from ox brain, purified, sequenced, synthesized, and characterized as antinociceptive in the rat (Yang et al. 1985). The two peptides, an octapeptide (F8Fa) and an octadecapeptide (A18Fa), have in common with FMRFamide the C-terminal dipeptide -Arg-Phe-NH_2 (Table 3) and are, like LPLRFamide, weakly active on molluscan bioassays for FMRFamide (Greenberg et al. 1987). In the rat, immunoreactivity to FMRFamide and the new bovine peptides is concentrated in the dorsal horn of the spinal cord and in the brain stem (Majane and Yang 1987); and some of the terminals in the spinal cord come from peripheral neurons (Ferrarese et al. 1986). Thus, these endogenous mammalian FaRPs may be interacting with opioid peptides at opioid receptors (Zhu and Raffa 1986) to modulate sensory input, particularly pain.

Pharmacology of FMRFamide in Vertebrates

FMRFamide has been tested on a variety of preparations, all but two of them from the rat; the observations are summarized in Table 4. Although many of the data represent opportunistic trials of a new agonist, some were more purposefully aimed. First of all, most of the behavioral experiments were meant to test the FMRFamide–opioid relationship, as described above. Second, the study of ON- and OFF-center double-color-opponent cells in the goldfish retina arose from the observation that retinal neurites originating in the nervus terminalis contained both FMRFamide- and LHRH-like immunoreactivity. This work (Stell et al. 1984; Walker and Stell 1986) is therefore pointed toward the functional role of colocalized putative peptide transmitters. Finally, the C-terminal dipeptide (-Arg-Tyr-NH_2) of the pancreatic peptide-related peptides (PP-RPs) is similar to that of FMRFamide, and antisera to FMRFamide crossreact weakly with the PP-RPs (reviewed by Greenberg et al. 1987). Thus, Sorenson et al. (1984) showed that FMRFamide immunoreactivity in the rat pancreas is localized in the PP-containing cells, and that FMRFamide (like pancreatic polypeptide) inhibits glucose-stimulated release of insulin and somatostatin from the perfused rat pancreas. In the end, they suggest "... that FMRFamide may be an economically useful alternative in studying the biological effects of the pancreatic polypeptide family of peptides" (Sorenson et al. 1984).

Summary

The attempt to extend the family of FMRFamide-related peptides to vertebrates led to the discovery of three, and potentially more, new neuropeptides in higher animals. Concomitantly, new information about the neuropeptide modulation of nociception was obtained. Finally, the effectiveness of FMRFamide as a vertebrate agonist suggests that it, or its analogs, might be useful tools in biomedical research.

A PROSPECT

The example of FMRFamide implies that other invertebrate neuropeptides will have analogs, not only in other invertebrate phyla, but also in the subphylum Vertebrata. Similarly, the pharmacological effectiveness of FMRFamide on vertebrate bioassay systems supports the notion that a limited number of analogous, complementary receptors are also widespread. Therefore, the discovery of new invertebrate neuropeptides, and then their vertebrate analogs, would have major biomedical significance. An interesting prospect for further investigation is described briefly below.

Echinoderm neuropeptides. The close phylogenetic relationship between the echinoderms and the vertebrate chordates is widely known. Yet due to major technical difficulties, the neurobiology of echinoderms has been studied only infrequently. In particular, no echinoderm peptides have ever been sequenced, although some interesting factors have been identified. For instance, the gonad stimulating substance extracted from isolated starfish nerves was identified as a peptide (Kanatani 1973), but never characterized. Possibly of greater general interest are neural factors that soften and stiffen the connective tissues of echinoderms. These factors appear to be peptides, but their chemistry has not been pursued (reviewed by Motokawa 1984). Echinoderms may represent a major untapped source of new peptide families.

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Marine Organisms as Models for the Study of Neuropharmacology

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INTRODUCTION

Certain marine organisms have served as useful materials for the study of neurophysiology and neuropharmacology for many years. These include giant axons of the squid and lobster, the walking leg nerves of the lobster, giant synapses of the squid, and giant neurons of *Aplysia*, to mention a few. In particular, squid giant axons have been used for voltage clamp analysis of ion channel gating kinetics, and represents the prototype from which a variety of modifications of the excitation scheme have been deduced. Much of our present knowledge of the mechanism of nerve excitation has been derived from the experimental data obtained with this preparation.

Aplysia neurons are also useful because of their large size and easy identification in the ganglia. The neurons have been used extensively for the analysis of behavior of neurons, and neural control of viscera and animal behavior. However, the use of *Aplysia* neurons for the study of neuropharmacology has been limited to some extent due to the difficulties in relating the data to humans.

This paper gives account of use of these marine organisms for the study of neuropharmacology. First, a brief history of development of squid and *Aplysia* preparations will be described. Second, a concise account of the mechanism of nerve excitation will be given as the basis on which neuropharmacological studies have been conducted. Third, a few examples of studies of drug–ion channel interactions using nerve preparations isolated from marine organisms will be given. An emphasis is placed on squid axon voltage clamp experiments with channel blockers such as tetrodotoxin, saxitoxin, and local anesthetics, and with channel modulators such as batrachotoxin, grayanotoxin, brevetoxins, palytoxin, and pyrethroids.

SQUID GIANT AXON

The history of the squid giant axon (diameter 400–800 μm) goes back to 1936 when J. Z. Young recognized that the large tubular structure emerging from the stellate ganglion, which had been thought to be a blood vessel, was an unusually large nerve fiber. However, it was not until the late 1930s and early 1940s that the squid giant axon was shown to be a truly remarkable nerve preparation. Curtis and Cole (1940) in the United States and Hodgkin and Huxley (1939) in Great Britain successfully recorded transmembrane resting potential and action potentials by inserting a glass capillary electrode longitudinally in the squid giant axon. The unusually large size of the squid giant axon also made it possible to carry out voltage clamp experiments, using a longitudinal glass capillary-axial wire electrode. Contrary to what had been believed at that time, based on the classical Bernstein's (1902, 1912) membrane hypothesis of resting and action potential generation, membrane potential was found to reverse polarity at the peak of the action potential. This overshoot formed the basis for development of the sodium theory of action potential generation first by Hodgkin and Katz (1949), who analyzed the action potential as a function of the external sodium concentration. This theory was later tested more thoroughly by using voltage clamp technique (Hodgkin and Huxley 1952*a, b, c, d*; Hodgkin et al. 1952).

Another remarkable development in using the squid giant axon occurred in 1961 when two groups succeeded in perfusing the axon intracellularly (Baker et al. 1961; Oikawa et al. 1961). Techniques used by the groups differed, but the principle was the same, i.e., to remove much of the axoplasm and to perfuse the empty axon with artificial solutions. This may sound like a relatively trivial development, but the impact on further advancement of knowledge was truly remarkable. Internal perfusion allows two very important experiments that are otherwise difficult to achieve. One is complete control of the internal environment, including ionic composition and pH. For instance, by replacing internal potassium with cesium, and by eliminating external potassium, the sodium current through the sodium channel can be recorded without contamination by potassium currents passing through any channels, and any ionic currents passing through the potassium channel. pH of the internal solution can be changed and controlled accurately. Many other manipulations can be achieved by changing internal media. The other advantage is the capability of applying any chemical to the internal phase at known concentrations. Some examples will be presented in this chapter.

SQUID GIANT SYNAPSE

A variety of synapse preparations have been used for electrophysiological studies. One of the most commonly used preparations is the isolated vertebrate neuromuscular junction. Although the physiology and pharmacology of the postsynaptic element (muscle) can be studied easily due to its large size, which permits microelectrode insertion, the presynaptic nerve terminal is too small for such manipulation. This precludes a variety of experimental protocols. There are a number of synapse preparations in ganglia and the brain, but small sizes of the synapses, especially those of presynaptic nerve terminals, make it almost impossible to apply sophisticated electrophysiological techniques, including voltage clamp. Giant synapses contained in the stellate ganglion of the squid meet many requirements for experimentation on the presynaptic element.

In the squid ganglion, fairly large presynaptic nerve fibers (40–70 μm in diameter) form axo-axonal synapses with a postsynaptic giant axon (ca. 500 μm in diameter) (Martin and Miledi 1986). The neurotransmitter is most likely l-glutamate. Due to their large diameters, it is possible to insert two glass capillary microelectrodes into each of the presynaptic and postsynaptic nerve fibers in the synaptic region. Thus a depolarizing current delivered to the nerve terminal generates a presynaptic action potential that, in turn, releases the transmitter. The postsynaptic response thereby produced can be recorded by a microelectrode inserted in the postsynaptic giant axon. It is also possible to

perform voltage clamp experiments with both presynaptic and postsynaptic fibers. This allows a variety of experiments. For instance, the relationship between the presynaptic depolarization and transmitter release can be measured by voltage clamping the presynaptic fiber while recording the postsynaptic response in the presence of TTX and TEA, which block sodium and potassium channels, respectively (Katz and Miledi 1967). The calcium channel current recorded from the presynaptic nerve terminal can be correlated with release of transmitter, which in turn generates the postsynaptic response (Llinás et al. 1981*a*, *b*).

Despite these advantages, use of squid giant synapse preparations for neuropharmacological studies has been limited for two reasons. One is difficulty in obtaining the material, which does not survive long in a laboratory. The preparation must be very fresh for the study of synaptic transmission that is highly vulnerable to deterioration. Therefore, it is almost necessary to work at a marine biological station where squid can be collected. The other reason is difficulty in extrapolating pharmacological data to mammals, which may respond to various drugs in a way different from the squid synapse. Furthermore, the squid synapse preparation is limited to glutamatergic transmission, and cannot be used for the study of drug action on other types of synapses, which are also important from the pharmacological point of view.

APLYSIA GIANT NEURON

It has been known for a long time that certain gastropod molluscs have unusually large nerve cells (Ihering 1877). However, it was not until the 1940s that giant neurons of the marine slug *Aplysia* and the land snail *Helix* proved to be useful preparations for electrophysiological experiments (Arvanitaki and Cardot 1941*a*, *b*, *c*, *d*). Because of their unusually large sizes and the limited number of giant neurons in each ganglion, it was possible to identify neurons in terms of the firing pattern and sensitivity to various neurotransmitters. Some fire spontaneously, while others are silent. Some respond to acetylcholine to produce either a hyperpolarization or a depolarization, while others respond to l-glutamate, 5-hydroxytryptamine, dopamine, or γ-aminobutyric acid (GABA). Extensive use of these giant neurons was reviewed by Tauc (1966) and Kandel (1976).

The large size of these neurons permits penetration by several microelectrodes without causing damage. For instance, one microelectrode can be used for potential recording, one for current delivery under voltage clamp conditions, and one for measuring internal ion activity. Internal perfusion can also be performed, making it possible to control internal environment. As with the squid giant axon, this is essential for precise voltage clamp measurement of a channel current. Since each neuron can easily be identified, *Aplysia* has also been used extensively for the study of neuronal control of animal behavior and of the activity of various internal organs (Kandel 1976).

In spite of these unique features, *Aplysia* giant neurons are not entirely satisfactory for use in the study of medical neuropharmacology. The main reason is that the properties of these molluscan neuroreceptors and ion channels are not necessarily the same as those of mammals. For the purpose of physiological and biophysical analyses this factor is less serious, but for the study of drug action it makes *Aplysia* and other invertebrate nerve preparations less desirable than mammalian preparations. Thus, invertebrate nerves have not enjoyed popularity as material for the study of neuropharmacology despite their convenient features including large sizes and easy availability.

One recent development in using *Aplysia* neurons deserves particular attention, i.e., application of tissue culture techniques. The use of cultured invertebrate neurons for the study of neurophysiology and neuropharmacology has so far been limited, and these preparations have only started receiving attention recently (see review by Townsel and Thomas 1987). Studies of neurotransmitter receptors in cultured *Aplysia* neurons (Bodmer and Levitan 1984), especially when combined with co-culture techniques (Camardo et al. 1983), are among the most promising for the application to neuropharmacology, albeit extrapolation of interpretation of data to humans is still problematical.

MECHANISM OF NERVE EXCITATION AND VOLTAGE CLAMP

Nerve excitation occurs as a result of opening and closing of ion channels in the membrane. Squid giant axons have been used extensively for this purpose. Figure 1 shows a schematic illustration of nerve excitation. In resting conditions, the nerve membrane is almost exclusively permeable to potassium. Therefore, the membrane potential value is close to the equilibrium potential for potassium (E_K = about −80 mV). This is because only potassium channels open and close at that large negative resting potential. When the membrane is stimulated by depolarization, the sodium channels open quickly. The potential of the membrane, now highly permeable to sodium, approaches the sodium equilibrium potential (E_{Na}), which is about +50 mV, generating the rising phase of an action potential. However, membrane depolarization also causes an inactivation of sodium channels, leading to their closing. Meanwhile, the potassium channels are opening slowly. Therefore, the membrane again becomes almost exclusively permeable to potassium, bringing the membrane potential close to the potassium equilibrium potential. These two events form the falling phase of the action potential.

Simple experiments to record the action potential do not permit measurements of channel opening and closing as described above. The only method that allows measurement of such events is the voltage clamp, in which membrane ionic currents can be recorded as a function of membrane potential and time. Two conditions are necessary for voltage clamping, one being "space clamp" and the other being "feed-back circuit." A schematic diagram of the voltage clamp is shown in Figure 2. A glass capillary electrode is inserted into a squid giant axon longitudinally, and the membrane potential is measured using a glass capillary reference electrode outside. The recorded membrane potential is amplified and fed into a control or feed-back amplifier to which command pulses are applied from a pulse generator. The difference between the recorded membrane potential and the command pulse is amplified by the control amplifier, and a current flows across the membrane via the internal axial wire electrode so that the membrane potential becomes equal to the command pulse. Thus the membrane potential is "clamped" at the command potential level, and the current necessary for this manipulation is recorded by a current recording amplifier. Under this condition, membrane potential

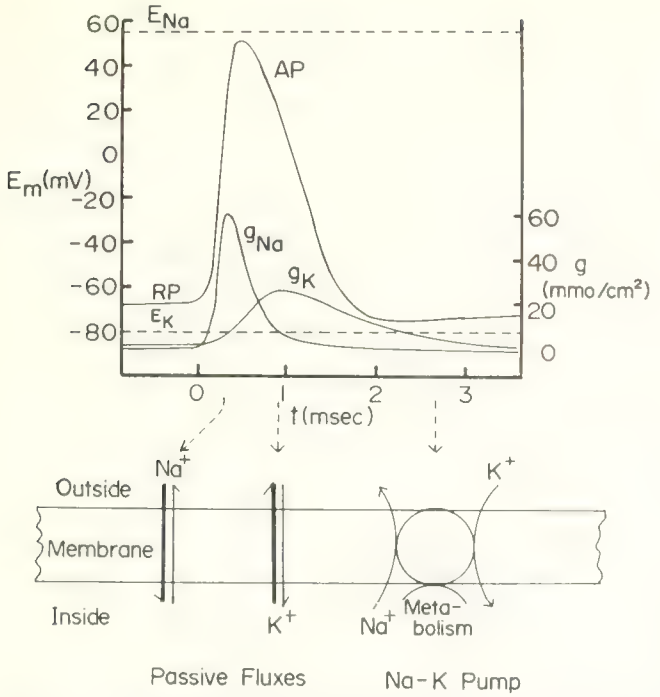

FIGURE 1. Mechanism of action potential generation. Upper half illustrates changes in membrane sodium conductance (g_{Na}) and potassium conductance (g_K) during an action potential (AP). Resting potential (RP) is close to the K^+ equilibrium potential (E_K), and the peak of the action potential approaches the Na^+ equilibrium potential (E_{Na}). Lower half illustrates ionic fluxes during the action potential and recovery. See text for further explanation. E_m, membrane potential; g, conductance. (From Narahashi 1984*b*.)

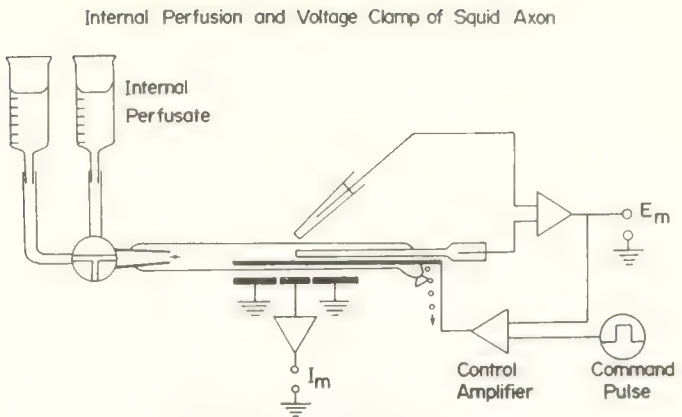

FIGURE 2. Voltage clamp of an internally perfused squid giant axon. See text for further explanation; E_m, membrane potential; I_m, membrane current. (From Narahashi 1984*b*.)

and membrane current can be measured, and membrane conductance can be calculated by Ohm's law. The axial wire electrode inside the axon and the large external current electrodes short-circuit the internal and external resistances, respectively, making it possible to keep the membrane potential and current distribution uniform along the portion of the axon where measurements are made.

Voltage clamp techniques are also applicable to various excitable cells other than squid giant axons. Different arrangements for electrodes are necessary to establish the space clamp condition. For various types of neurons three methods have been used, depending on the size of neuron and on the current signals to be measured. Two-microelectrode voltage clamp techniques can be applied effectively to relatively large neurons with a diameter of about 50 μm or more. Two glass capillary microelectrodes are inserted in a neuron, one serving for potential measurement and the other for current delivery. A few examples among many include *Aplysia* neurons (Lewis and Wilson 1982) and puffer fish neurons (Hagiwara and Saito 1959). A second method is to have one intracellular microelectrode for both potential recording and current delivery, using an electronic switch (Wilson and Goldner 1975). However, this technique does not permit measurement of high frequency responses, and therefore is not suitable for fast currents such as those of sodium. A third method is to use a patch electrode as described below. This technique is very versatile; it can be applied to small cells and combined with intracellular perfusion (Hamill et al. 1981).

The current record associated with a step depolarizing pulse under voltage clamp conditions is illustrated in Figure 3. Upon step depolarization, a brief, large, outward current flows. This is a capacitive current, and is followed by a transient inward current carried by sodium ions. The sodium current decays slowly and is followed by a steady-state outward current carried by potassium ions. Thus the peak transient current represents the sodium current, and the steady-state current represents the potassium current. The membrane conductances to sodium and potassium (g_{Na} and g_K) can be calculated by the following equations, where I_{Na} and I_K refer to sodium and potassium currents, respectively, and E_m refers to the membrane potential:

$$g_{Na} = I_{Na}/(E_m - E_{Na})$$
$$g_K = I_K/(E_m - E_K)$$

The classical voltage clamp experiments described above do not measure the activity of individual ion channels. The membrane ionic current thus recorded represents an algebraic sum of ionic currents passing through a large number of ion channels present in the membrane where measurements are made. Development of patch clamp techniques, first introduced by Neher and Sakmann (1976), and later improved by Hamill et al. (1981), revolutionized ion channel study, because it is now possible to measure ionic currents passing through individual ion channels. There are at least two requirements for successful patch clamp single channel recording experiments. First, the channel density should be low enough to record individual channel opening and

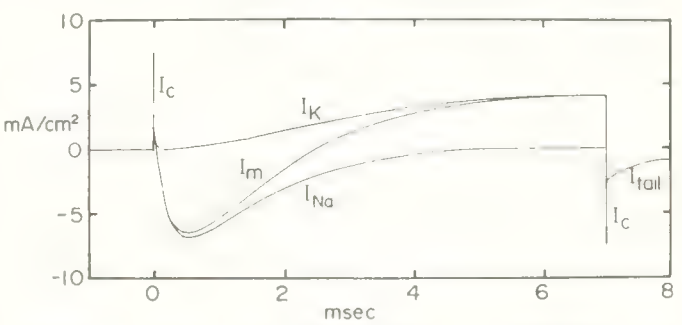

FIGURE 3. Membrane current associated with a step depolarization of the membrane under voltage clamp conditions. I_c, capacitive current; I_K, potassium current; I_m, membrane (total ionic) current; I_{Na}, sodium current; I_{tail}, tail current. (From Narahashi 1981.)

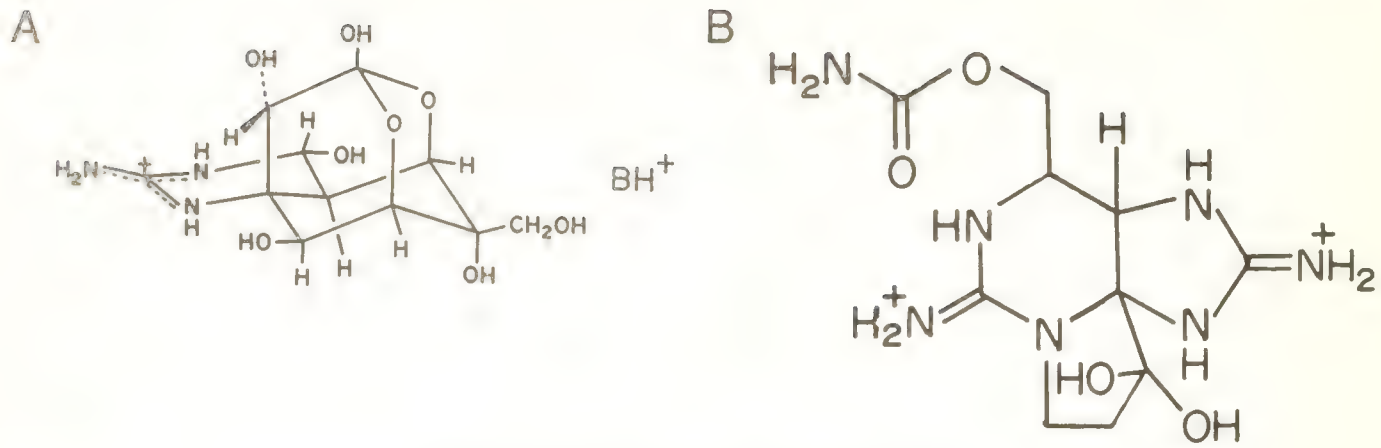

FIGURE 4. Structures of tetrodotoxin (A) and saxitoxin (B).

closing separately. Second, the membrane surface should be free of other tissues such as connective tissues and Schwann cells so that a "gigaohm seal" around the rim of the capillary electrode tip can be formed. Giant axons are difficult to handle for patch clamping, but many other preparations have been successfully used including cultured neurons and myocytes, gland cells, denervated skeletal muscles, and lymphocytes.

Patch clamp techniques can also be used to record membrane ionic currents from whole cells (Hamill et al. 1981). The whole cell patch clamp techniques offer extremely broad applicability to practically any type of cells unless the cell diameter is very small.

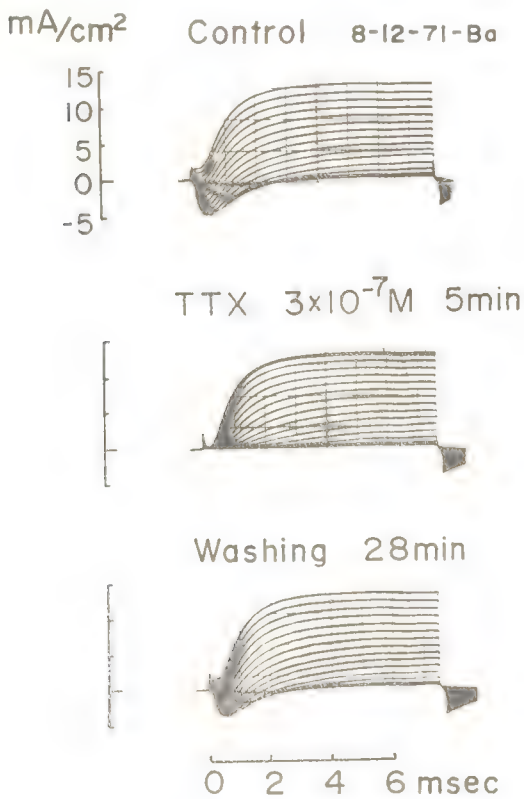

FIGURE 5. Families of membrane currents associated with step depolarizations (10 mV steps) in a squid giant axon before and during external applications of 300 nM tetrodotoxin (TTX) and after washing with toxin-free medium. Note that TTX blocks transient sodium currents without any effect on steady-state potassium currents. (From Narahashi 1975.)

PHARMACOLOGICAL AND TOXICOLOGICAL STUDIES

A number of studies have been performed using nerve preparations of marine organisms, especially squid giant axons, to elucidate the mechanisms of action of drugs, toxins, and other chemicals on ion channels. This chapter is limited to a few examples of such studies to illustrate the usefulness of these preparations. Therefore, details are omitted, and those interested in more information are advised to refer to cited papers.

Chemicals acting on ion channels may be divided into two large categories based on the type of action (Narahashi 1984*a*). These are channel blockers and channel modulators. Channel blockers are chemicals that eliminate ionic currents. They do not necessarily impair the channel gating machinery but may simply occlude the channel. However, other types of blockers eliminate ionic currents by impairing or inhibiting the gating machinery. Channel modulators are chemicals that alter the kinetics of gating machinery. Some modulators slow the rate at which the sodium inactivation (h) gate is closed, causing a prolonged sodium current. Some others modify the rate at which the sodium activation (m) gate functions, also causing a slowing of sodium current.

A. Channel Blockers

1. Tetrodotoxin and Saxitoxin

Tetrodotoxin (TTX) (Fig. 4) is a potent neuropoison contained in the liver and ovary of pufferfish. Since the discovery of its highly potent and specific action blocking the nerve membrane sodium channel (Narahashi et al. 1960, 1964) (Fig. 5), TTX has become a very useful, popular chemical tool for the study of neurophysiology and neuropharmacology (see Narahashi 1974, 1987; Catterall 1980; Pappone and Cahalan 1986). Saxitoxin (STX) (Fig. 4) is contained in toxic dinoflagellates such as *Gonyaulax catanella*, and exerts the same effects as TTX (Narahashi et al. 1967). STX has also been used as a tool.

Axons exposed to TTX or STX can be used to record the potassium current without contamination by the sodium cur-

TABLE 1. SODIUM CHANNEL DENSITIES ESTIMATED FROM TTX AND STX BINDING.

Preparation	Method	Density per μm^2	Reference
Lobster walking leg nerve	TTX bioassay	<13	Moore et al. (1967)
Lobster walking leg nerve	[^{3}H]-STX binding	90	Ritchie et al. (1976)
Garfish olfactory nerve	[^{3}H]-STX binding	35	Ritchie et al. (1976)
Squid giant axon	[^{3}H]-STX binding	290	Keynes and Ritchie (1984)
Rabbit vagus nerve	[^{3}H]-STX binding	110	Ritchie et al. (1976)
Rabbit sciatic node of Ranvier	[^{3}H]-STX binding	12,000	Ritchie and Rogart (1977*b*)
Mouse neuroblastoma cell	[^{3}H]-STX binding	78	Catterall and Morrow (1978)
Frog sartorius muscle	[^{3}H]-STX binding	195–341	Ritchie and Rogart (1977*a*)
Rat diaphragm muscle	[^{3}H]-STX binding	421	Bay and Strichartz (1980)

rent. The toxins and their tritiated forms have been used to measure binding to the sodium channel site, from which sodium channel density was estimated. The first such study was conducted by us with the lobster walking leg nerve. This preparation contains a large number of relatively small nerve fibers, and therefore the total membrane area is very large. Using a TTX bioassay method, the sodium channel density was estimated to be a maximum of 13 per square μm of membrane (Moore et al. 1967). Later, several other investigators used tritiated TTX or STX, and more accurate estimates of the sodium channel density are now available; some are listed in Table 1. With the exception of the node of Ranvier, sodium channels are sparsely distributed in nerve membranes.

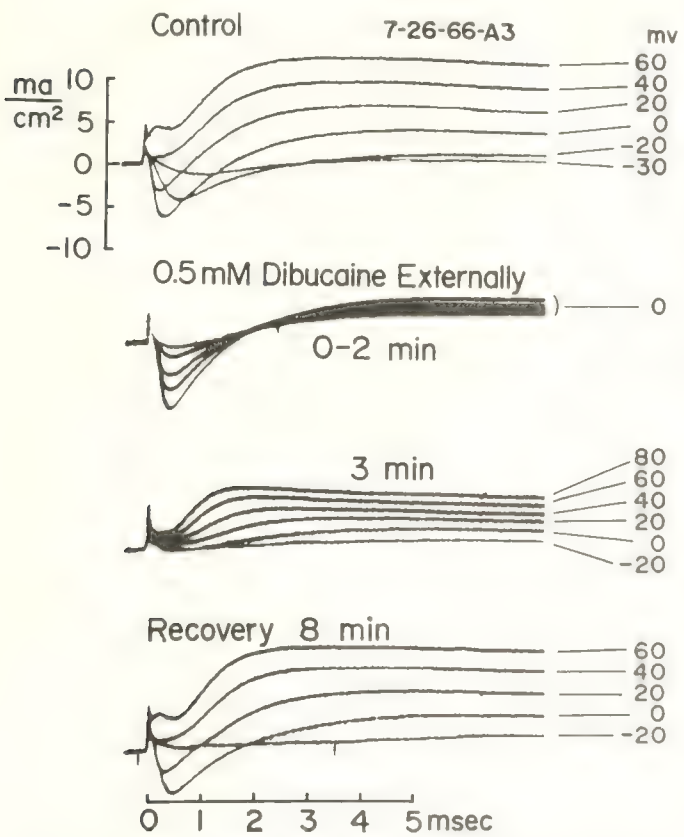

FIGURE 6. Suppression of both Na and K currents by externally applied 0.5 mM dibucaine to a squid giant axon. Top records are families of normal membrane currents associated with step depolarizations to the levels indicated. Second records represent changes in currents at the potentials indicated during the first 2 min of drug application. Third records are families of membrane currents in the presence of drugs. Bottom records show recovery after washing. (From Narahashi et al. 1969.)

The toxins have also been used to identify presence or absence of sodium channels in a tissue. In certain *Aplysia* or *Helix* neurons, action potentials are generated as a result of opening of both sodium and calcium channels (Geduldig and Junge 1968). Application of TTX to such neurons, or removal of sodium from external perfusate, suppresses but does not completely block the action potential because only the component of the action potential generated by the sodium channel is blocked, disclosing the calcium action potential. The sodium action potential can be observed by application of cobalt ions, a calcium channel blocker, or by removal of calcium ions from the external perfusate.

TTX and STX have no effect on transmitter release from the nerve terminals nor on the postsynaptic membrane, so toxin-treated synapse preparations have been used for the study of the mechanism of transmitter release without complication by action potentials. In experiments with the squid giant synapse preparation, TTX was added to the external perfusate while tetraethylammonium (TEA), a potassium channel blocker, was injected into the presynaptic nerve fiber. No action potential could be generated in either presynaptic or postsynaptic fibers, yet a postsynaptic depolarizing response could be produced when the neurotransmitter was released by applying a depolarizing pulse to the presynaptic nerve terminal (Katz and Miledi 1967).

The mechanism of sodium channel block by TTX and STX has been well characterized (see Narahashi 1974, in press). TTX and STX selectively block the sodium channel at or near its external opening without modifying the gating mechanism. TTX is effective only when applied outside of the membrane. The gating current remains unchanged while the sodium current is blocked. Thus, in the presence of TTX, the channel gating machinery works normally but the channel is occluded at or near the external mouth (Kao and Nishiyama 1965; Hille 1975; Kao 1983). It has indeed been demonstrated that TTX blocks the

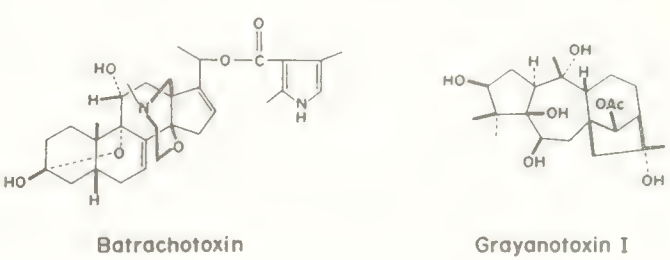

FIG. 7. Structures of batrachotoxin and grayanotoxin I.

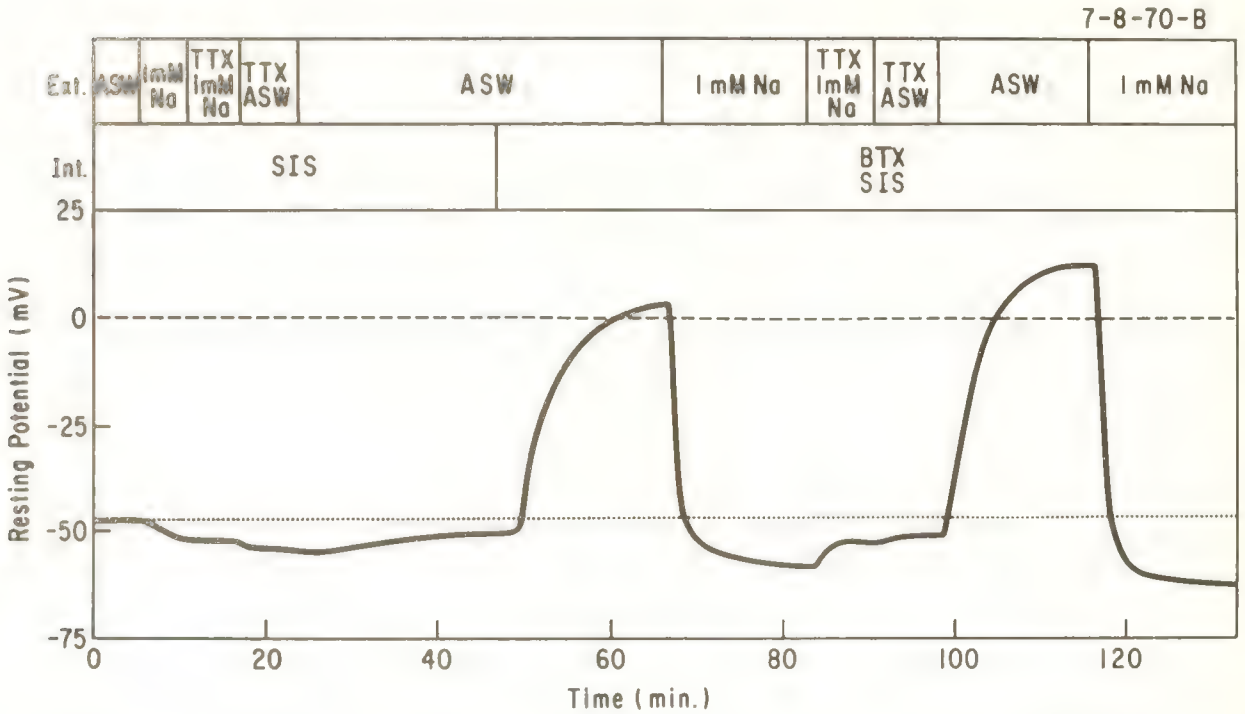

FIGURE 8 Effects on the resting membrane potential of an internally perfused squid giant axon of: 1) 550 nM batrachotoxin (BTX) applied internally; 2) 1,000 nM tetrodotoxin (TTX) applied externally; and 3) 1 mM Na applied externally. Standard internal solution (SIS) contains 50 mM Na. External medium is ASW (artificial seawater). (From Narahashi et al. 1971.)

sodium channel on a 1:1 stoichiometric basis by dose-response curve analyses (Cuervo and Adelman 1970), and by single channel recording experiments (Quandt et al. 1985).

2. Local Anesthetics

Squid giant axons have proven to be useful preparations for the study of local anesthetic action. In fact, the first pharmacological study using the voltage clamped squid axon was conducted for this purpose (Shanes et al. 1959; Taylor 1959). Both sodium and potassium currents are suppressed by all local anesthetics so far examined (e.g., Fig. 6). Most local anesthetics used clinically are tertiary amines with a pK_a of 7–9, so they exist in the charged cationic form and the uncharged molecular form. Questions as to active form and site of action were addressed by experiments using internally perfused squid giant axons (Frazier et al. 1970, 1971; Narahashi et al. 1970). In short, local anesthetic molecules penetrate the nerve membrane in the uncharged form, are ionized in the axoplasm, and block the sodium and potassium channels from inside in the cationic form. The binding site of the local anesthetic molecule is located within the sodium channel (Hille 1977).

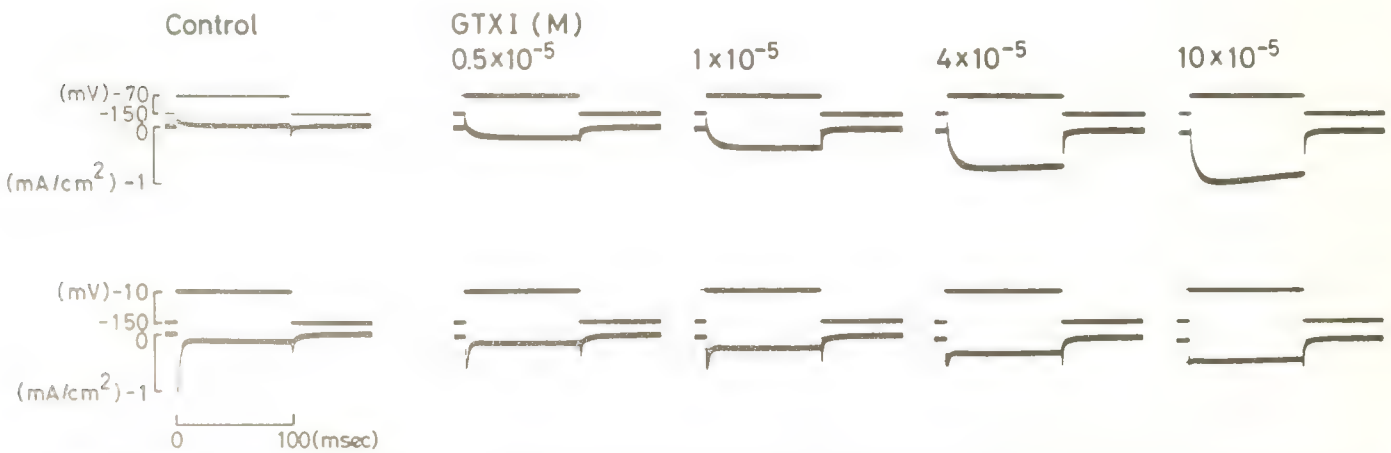

FIGURE 9 Squid giant axon membrane Na currents associated with step depolarizations from the holding potential of −150 mV to −70 mV (upper diagram) and to −10 mV (lower diagram) before and during internal perfusion with various concentrations of grayanotoxin I (GTX I). This was done in the presence of 20 mM tetraethylammonium inside the cell to block the K channels. At −70 mV only slow Na currents are generated in the presence of grayanotoxin I, and at −10 mV the peak transient Na currents are followed by slow Na currents in the presence of grayanotoxin. (From Seyama and Narahashi 1981.)

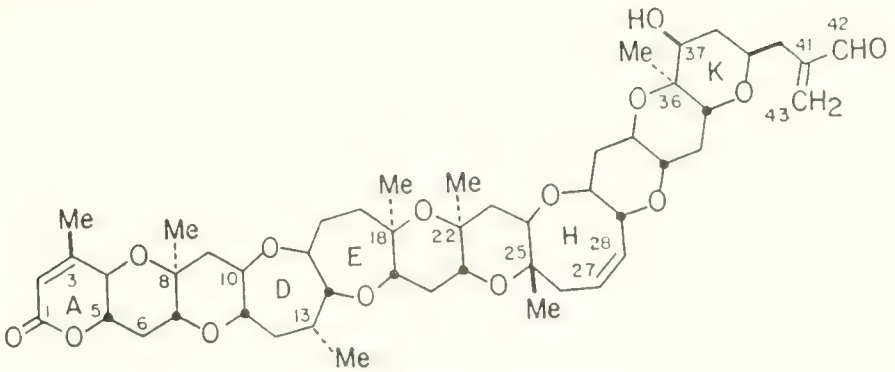

FIGURE 10. Structure of brevetoxin B.

B. CHANNEL MODULATORS

Certain chemicals and toxins have been found to modulate gating kinetics of ion channels. The sodium channel has activation (m) and inactivation (h) gates, one or both of which could be modified by a chemical. Therefore, the mechanism underlying channel modulation could be very complex. Only a few examples will be described to illustrate the situation.

1. Batrachotoxin and Grayanotoxin

Batrachotoxin (BTX) is contained in the skin secretion of the Colombian arrow poison frog *Phyllobates aurotaenia*. It has a steroidal structure (Fig. 7), and is a very potent nerve and muscle poison with an LD_{50} of about 1 μg/kg. Grayanotoxin (GTX) (Fig. 7) is the toxic principle contained in the leaves of various plants (e.g., *Leucothoe, Rhododendron, Andromeda, Kalmia*) that belong to the family Ericaceae. Both toxins exert similar effects on nerve membrane sodium channels, keeping them open for an unusually long period, allowing a prolonged sodium current (Narahashi 1974, 1984*a*). They are effective from either side of the membrane.

One classical experiment using the squid giant axon for the study of BTX action is illustrated in Figure 8. When perfused internally, BTX causes a large depolarization of the membrane that is reversed in polarity. The depolarization disappears and the membrane even hyperpolarizes slightly by reducing the external sodium concentration from the normal value of 450 mM to 1 mM. TTX also antagonizes BTX-induced repolarization. In the absence of sodium in both external and internal phases, BTX exerts no effect on the membrane potential. Similar results have been obtained with GTX. These observations clearly indicate that BTX and GTX keep the sodium channels open for a long time. Single sodium channel recording experiments with cultured neuroblastoma cells have demonstrated that individual channels are kept open for a long time in the presence of BTX, and that the BTX-modified channels can open at large negative potentials when normal sodium channels cannot open (Quandt and Narahashi 1982). These two changes in single channel kinetics account for the observed membrane depolarization.

As predicted from these data on BTX and GTX modulation of sodium channels, the kinetics of the sodium current recorded from the whole cell or the giant axon undergo drastic changes after exposure to the toxins (Fig. 9). The sodium current can be generated at large negative potentials (e.g., −80 mV), and the current is maintained at a steady-state level during a depolarizing step or the sodium inactivation mechanism is totally impaired (Seyama and Narahashi 1981; Tanguy et al. 1984).

2. Brevetoxins

Brevetoxins, contained in the dinoflagellate *Ptychodiscus brevis*, are known to cause hyperexcitability of the nervous system, including repetitive discharges and an increase in transmitter release from the nerve terminals (Wu et al. 1985). There are several components in brevetoxins. The structure of brevetoxin B is shown in Figure 10. Their effects on the membrane potential

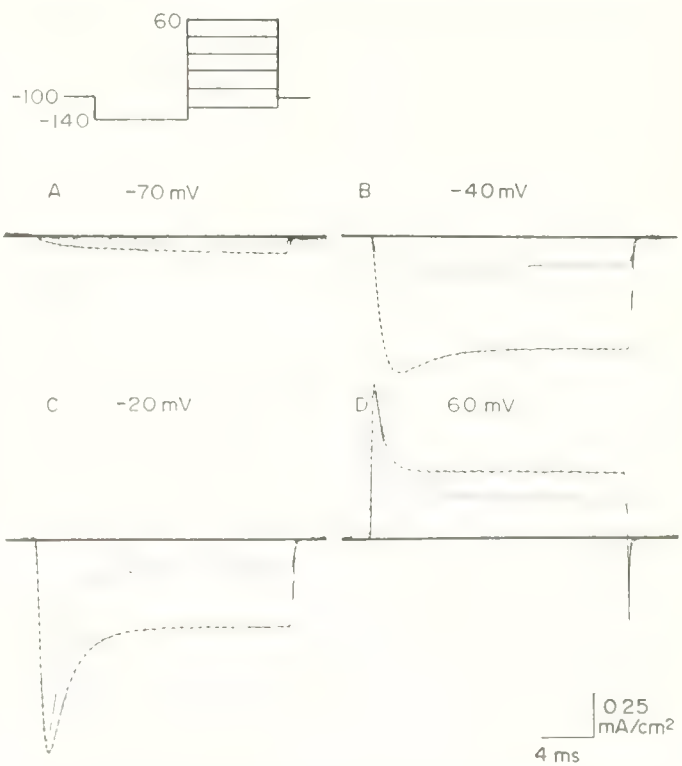

FIGURE 11. Effects of internally applied 30 μM brevetoxin-B on Na currents recorded from a squid giant axon. Sodium currents during 20 mV step depolarizations to −70, −40, −20, and +60 mV are shown. A 20 ms prehyperpolarization to −140 mV was applied from the holding potential of −100 mV before the test step depolarizations (inset). Each of panels a–d shows superimposed records of control and brevetoxin-B-treated axons. The toxin caused both peak and steady-state sodium current to increase. Na concentrations in external and internal perfusates were 100 mM and 50 mM, respectively. (From Atchison et al. 1986.)

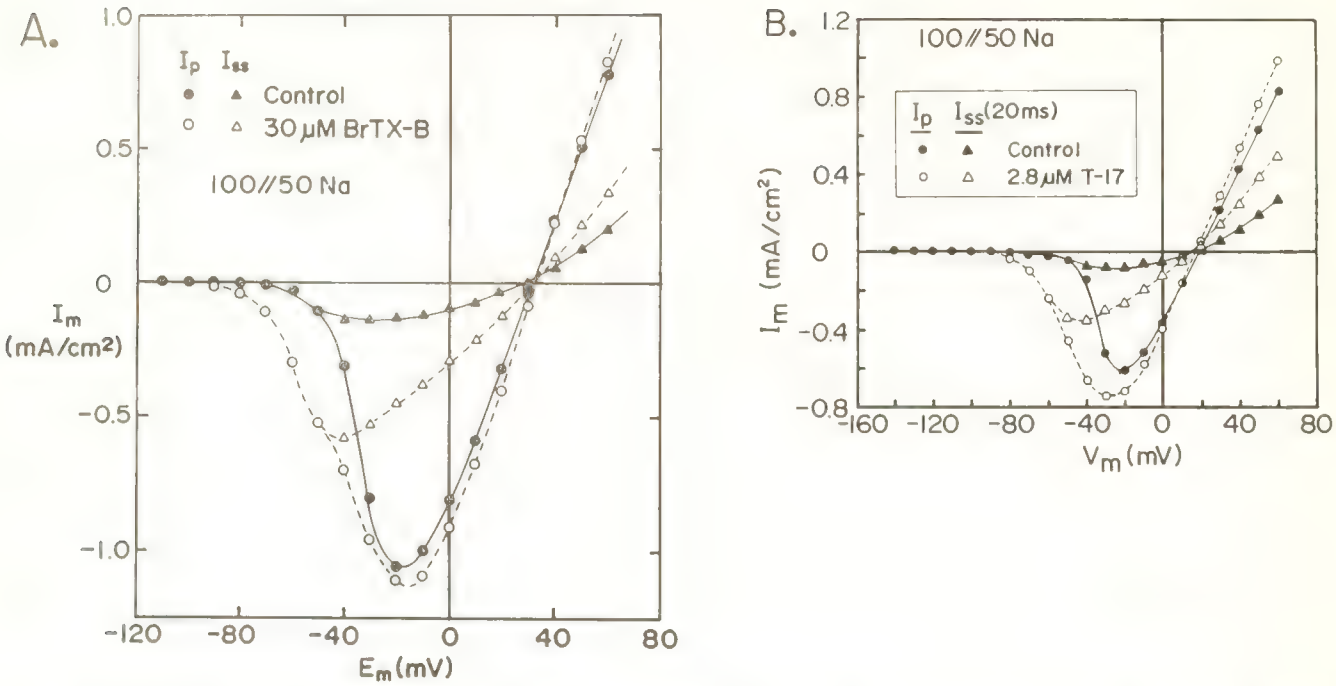

FIGURE 12. Current–voltage (I_m–E_m) relationships for the peak (I_p) and steady-state (I_{ss}) currents in the absence (filled symbols) and presence (open symbols) of 30 μM brevetoxin-B (BrTX-B) (a) or 2.8 μM T-17 toxin (b). Data obtained using the protocol for Figure 11. External and internal Na concentrations were 100 mM and 50 mM, respectively. (From Atchison et al. 1986.)

of squid axons are similar to those of BTX. Either external or internal application is effective. The membrane is depolarized, and the effect is reversed by lowering the external sodium concentration or by external application of TTX (Huang et al. 1984; Wu et al. 1985). As predicted, brevetoxins greatly increase the frequency of spontaneous miniature end-plate potentials, and the effect is antagonized by TTX (Atchison et al. 1986). Voltage clamp experiments with squid giant axons have revealed that the peak transient sodium current are both increased by brevetoxins (Fig. 11). Current–voltage relationships of peak and steady-state sodium currents clearly show that the curves are shifted in the direction of hyperpolarization after application of brevetoxins (Atchison et al. 1986). This means that the sodium channel can be opened at large negative potentials where normally channels do not open (Fig. 12).

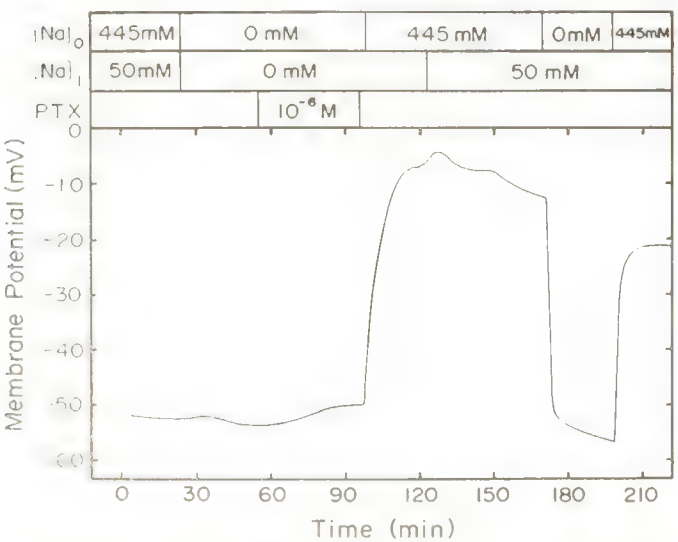

FIGURE 13. Absence of PTX depolarization in Na-free external and internal media. PTX (1 μM) caused only a negligible depolarization when external and internal solutions were devoid of sodium, but a large and rapid depolarization ensued as soon as the normal sodium concentration (445 mM) was restored in the external solution. Removal of sodium from the external solution in the presence of 50 mM Na inside caused a hyperpolarization beyond the level of the original resting potential. $[Na]_o$ and $[Na]_i$ refer to the external and internal sodium concentrations, respectively. (From Muramatsu et al. 1984.)

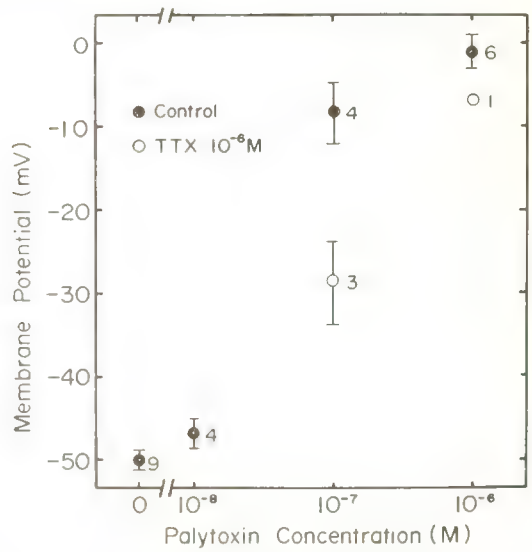

FIGURE 14. Dose–response relationships for the depolarizing action of PTX with and without 1 μM TTX. The depolarization was antagonized by TTX to only a limited extent. Data are given as the mean ± SEM, along with number of experiments. (From Muramatsu et al. 1984.)

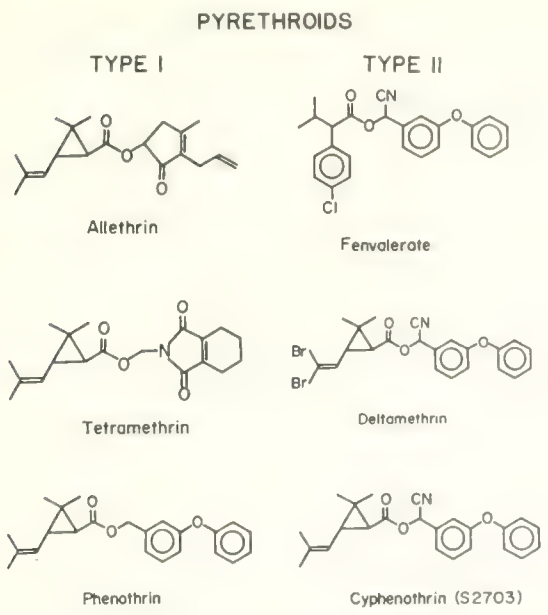

FIGURE 15. Structures of type I and type II pyrethroids. (From Narahashi 1985.)

3. Palytoxin

Palytoxin (PTX) is the toxic component isolated from various species of the zoanthid *Palythoa*, and is one of the most potent toxic substances known (see Muramatsu et al. 1984). The LD_{50} value was estimated to be 0.15 μg/kg in mice by i.v. injection, making PTX some 60 times more toxic than TTX. The chemical structure of PTX isolated from *Palythoa tuberculosa* has been identified (Moore and Bartolini 1981; Uemura et al. 1981). Its molecular formula is $C_{129}H_{220}N_3O_{54}$, and it has a molecular weight of 2,680 daltons.

PTX causes a large membrane depolarization in the squid giant axon by a unique mechanism (Muramatsu et al. 1984). It is effective only from outside the membrane. Depolarization can be observed even at 10 nM, and is reversed very slowly during prolonged washing with toxin-free media. The depolarization is sodium-dependent: reducing external sodium concentration from the normal level of 445 mM to 1 mM abolishes the PTX-induced depolarization, and no depolarization is observed in the absence of sodium in both external and internal phases (Fig. 13). Thus the large depolarization caused by PTX is due to an increase in sodium permeability of the membrane.

Contrary to BTX or GTX, PTX-induced depolarization is not effectively reversed by TTX (Muramatsu et al. 1984). Figure 14 shows the dose–response relationships of PTX-induced depolarization in the presence and absence of 1 μM TTX. TTX only slightly restores the PTX-induced depolarization.

These experiments suggest that the sodium permeability increase caused by PTX is due not to opening sodium channels but to some other mechanism. Relative permeabilities to various cations have been measured to characterize the PTX-induced sodium permeability increase (Muramatsu et al. 1984). The permeability of test cation (P_X) relative to the sodium permeability (P_{Na}) is estimated to be 1:0.62:0.75:1.45 for P_{Na}:P_{Li}:P_{Cs}:$P_{ammonium}$. The equivalent value for normal squid axon is 1:1.12:0.028:0.212 (Hironaka and Narahashi 1977; Seyama and Narahashi 1981). Thus the PTX-induced ionic permeability is considerably different from that of the normal sodium channel. The voltage dependence of peak sodium current and steady-state potassium current is greatly shifted in the hyperpolarizing direction by application of PTX, yet the kinetics of sodium current remain unchanged, albeit there is a slight decrease in amplitude. These results may be interpreted as being due to the creation of a new channel in the membrane by PTX.

4. Pyrethroids

Pyrethroids are synthetic derivatives of natural pyrethrins, which are contained in the flowers of *Chrysanthemum cinerariaefolium*. The flowers were used widely as insecticide until the end of World War II, but have been largely displaced by synthetic insecticides such as DDT, lindane, parathion, malation, and dieldrin. However, serious environmental concerns over the long-lasting toxic action of synthetic insecticides revived interest in pyrethrins. A large number of pyrethroids have

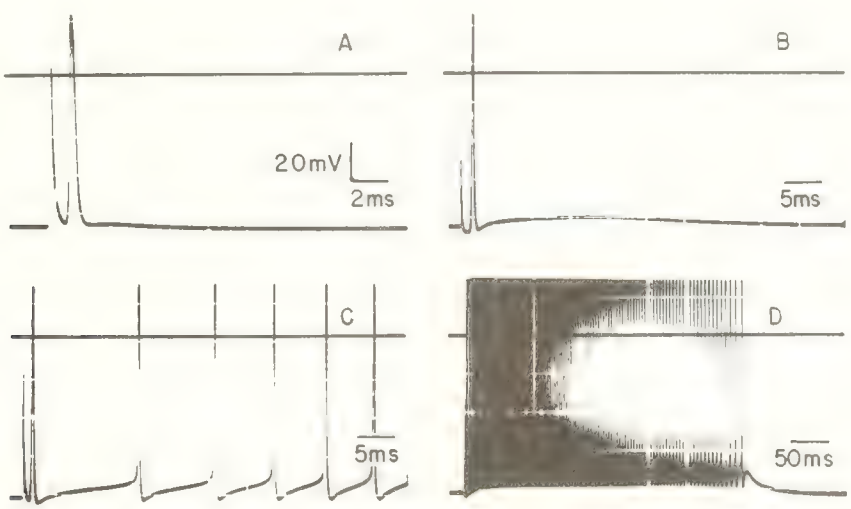

FIGURE 16. Repetitive discharges induced by a single stimulus in a crayfish giant axon exposed to 10 μM (+)-trans tetramethrin. Intracellular recording at 22°C. A, control; B, 5 min after application of tetramethrin; C and D, after 10 min. (From Lund and Narahashi 1981*a*.)

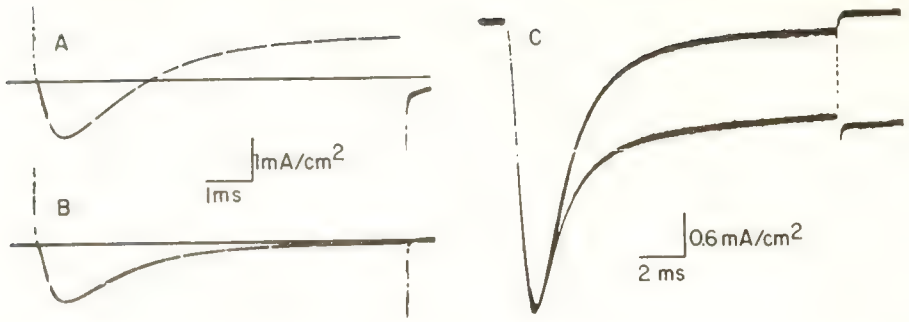

FIGURE 17. Membrane currents associated with step depolarizations in a squid giant axon before and during internal perfusion with 1 μM (+)-trans allethrin. (A) Peak inward Na current followed by steady-state outward K current when the membrane is step depolarized from −80 mV to 0 mV. (B) Na current after Cs was substituted for K^+ in the internal perfusate, and tetramethylammonium was substituted for K^+ in the external perfusate to eliminate the K current. (C) Na current associated with step depolarization from −100 mV to −20 mV in the K^+-free medium before (current with a small residual component, upper recording) and during (current with a large residual component, lower recording) internal perfusion with allethrin. (From Narahashi 1984*b*.)

been synthesized and tested for insecticidal activity and mammalian toxicity in the 1960s and 1970s. Some have proven very useful, safe, and biodegradable insecticides, and are now used extensively.

Pyrethroids are esters that can be divided into two structural groups (Fig. 15). The pyrethroids that lack the cyano group at the α position are called type I, and include allethrin, tetramethrin, phenothrin, and permethrin. Those that contain the α cyano group are called type II, and are represented by deltamethrin, cyphenothrin, cypermethrin, and fenvalerate. The symptoms of poisoning in mammals are somewhat different in the two types. Symptoms caused by type I pyrethroids are hyperexcitation, ataxia, convulsions, and paralysis. Type II pyrethroids cause hypersensitivity, choreoathetosis, tremors, and paralysis (Narahashi 1985).

Squid giant axons are very useful for the study of pyrethroids (Narahashi and Anderson 1967; Wang et al. 1972; Starkus and Narahashi 1978; Lund and Narahashi 1981*b*, 1982). The sodium channel has been clearly demonstrated to be the major target site of pyrethroids, and poisoning symptoms can be accounted for on this basis (see reviews by Narahashi 1985; Ruigt 1984).

a. *Type I pyrethroids.* Allethrin and tetramethrin cause repetitive discharges of the squid giant axon. In normal preparations, a single electrical stimulus generates one action potential. After external or internal exposure to the pyrethroids, a single stimulus produces repetitive after-discharges as a result of an increase in depolarizing after-potential that follows the spike (Fig. 16). Voltage clamp experiments have shown that the increase in depolarization is due to a prolonged flow of sodium current (Narahashi and Anderson 1967; Lund and Narahashi 1981*a*, *b*). Prolonged sodium current flows even after termination of a depolarizing pulse. In a normal axon, only a small "tail current" flows upon step repolarization of the membrane, but in the poisoned axon the tail current is greatly increased in initial amplitude and decays very slowly (Fig. 17). This indicates changes in the activation (m) kinetics.

The prolonged sodium current is due to prolonged opening of individual sodium channels, as revealed by patch clamp experiments with neuroblastoma cells (Yamamoto et al. 1983). Amplitude of single channel current is not affected by pyrethroids. When affected by pyrethroids, individual sodium channels can open during a prolonged depolarizing pulse, a situation

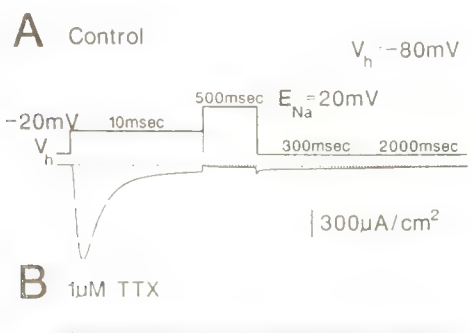

FIGURE 18. Sodium current from a control squid axon before (A) and after (B) external application of 1 μM TTX. External and internal sodium concentrations were 111 mM and 50 mM, respectively. A. A depolarizing pulse from the holding potential (V_h) of −80 mV to −20 mV elicited the normal transient inward sodium current, which decayed within 10 msec. Depolarization to a second depolarizing pulse (500 msec) to the sodium reversal potential (E_{Na} = +20 mV) yielded a negligible current. Repolarization to the holding potential (−80 mV) produced a very small inward sodium tail current. B. All currents were blocked by 1 μM TTX in the external solution. The pulse protocol was the same as that described in A. (From Brown and Narahashi 1987.)

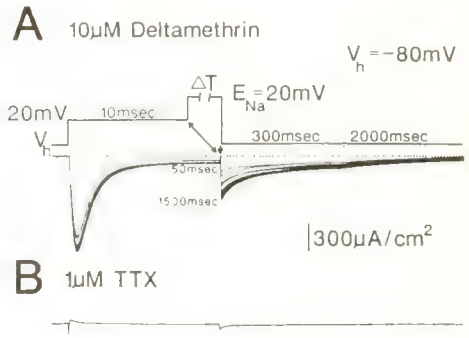

FIGURE 19. Sodium current from a squid axon perfused internally with 10 μM deltamethrin. External and internal sodium concentrations were 111 mM and 50 mM, respectively. A depolarizing pulse from the holding potential (V_h) of −80 mV to −20 mV elicited a transient inward sodium current which decayed within 10 msec. Steady-state current at the end of this pulse was increased. Repolarization to the holding potential from a second depolarizing pulse to the sodium reversal potential (E_{Na} = +20 mV) yielded a large inward sodium tail current which decays very slowly. The amplitude of this tail current increased with increasing pulse duration. B. After application of 1 μM TTX in the external solution, all currents were blocked. The same protocol was used as described in A. (From Brown and Narahashi 1987.)

not seen in normal preparations. This indicates changes in inactivation (h) kinetics.

A new concept of toxicological amplification has been developed as a result of pyrethroid experiments (Lund and Narahashi 1982). Less than 1% of the sodium channel population must be modified by pyrethroids to increase the depolarizing after-potential to the threshold level for induction of repetitive discharges. This means that the effect of pyrethroids on a limited number of sodium channels is amplified, through the threshold phenomenon involving increase in depolarizing after-potential, to bring about repetitive discharges, which in turn cause the symptoms of poisoning in mammals and insects.

b. *Type II pyrethroids.* Although the major target site of type II pyrethroids is also the sodium channel, the kinetic changes are different from those caused by type I pyrethroids. The sodium current recorded from the squid giant axon is prolonged in the presence of deltamethrin (Fig. 18, 19), a type II pyrethroid (Brown and Narahashi 1987). The time course of decay of the tail current is much slower than that observed in axons poisoned by type I pyrethroids. Because of much slower decay of the sodium current, the membrane is depolarized. Single channel recording experiments have revealed a marked prolongation of open time, sometimes reaching as long as several seconds from the control value of a few milliseconds (Holloway et al. 1984; Chinn and Narahashi 1986).

Due to membrane depolarization, sensory neurons are stimulated to increase discharge frequency, resulting in a tingling sensation of the face of persons exposed to the type II pyrethroids. Membrane depolarization will also cause transmitter release from the nerve terminals, resulting in disturbance of synaptic transmission. Thus the nervous system function as a whole will be affected in a way different from that caused by type I pyrethroids.

CONCLUSION

A variety of nerve preparations isolated from marine organisms have proven very useful materials for the study of neuropharmacology. In particular, the squid giant axon has been used extensively, and much of the present knowledge of nerve excitation mechanisms is deduced from experiments using it. Other marine preparations used for such study include *Aplysia* giant neurons and squid giant synapses. These preparations will continue to be useful for neuropharmacology because their large sizes permit highly precise measurements of various membrane properties. The only reservation that must be made is the fact that invertebrate nerve preparations may be different from mammalian nerves in their pharmacological and physiological characteristics. Therefore, the most efficient way of using marine nerve preparations would be to take advantage of their large sizes and to analyze the detailed mechanisms underlying the drug–channel interaction that represents a common denominator between the invertebrate and mammalian nerve preparations.

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Use of Selective Toxins to Examine Acetylcholine Receptor Structure

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INTRODUCTION

Many biological toxins have evolved to achieve the requisite specificity and thus can serve as invaluable tools for the study of both structure and function of cell surface receptors. Several examples of these toxins and the receptors they interact with have been presented in this volume. Perhaps the most widely studied receptor, and the one in which our knowledge of structure and function remains the most advanced, is the nicotinic acetylcholine receptor. In addition to playing a role in the autonomic and central nervous systems, this receptor is found at postsynaptic endplates of skeletal muscle and is critical to neuromuscular transmission for all voluntary motor activity of vertebrates (Taylor 1985).

The recognition that natural plant alkaloids would block neuromuscular transmission actually preceded the concept of receptors. In fact, the pioneering work of Claude Bernard documented in the 1850s the locus of action of the Southern American arrow poison, tube curare, the purified alkaloid of which is now known as d-tubocurarine. It was Bernard who also clearly pointed out the value of such pharmacologic or toxic agents in delineating sites of physiologic actions (Bernard 1856). d-Tubocurarine, related plant alkaloids, and their synthetic congeners continue to be employed clinically as muscle relaxants. Antagonism by d-tubocurarine can be shown to be competitive with the natural agonist acetylcholine, thus it is likely that its binding surface overlaps that of agonists. The plant alkaloids that antagonize the receptor contain tertiary or quaternary nitrogens that are positively charged at physiological pH (Fig. 1). More than a century after Bernard's experiments, Chang and Lee (1963) showed that venom of cobra and certain sea snakes contains peptide α-toxins of 6,500–7,500 daltons that block with apparent irreversibility at the post synaptic surface of the neuromuscular junction (Chang and Lee 1963). This apparent irreversibility does not appear to be due to covalent binding but rather to the toxin's high affinity and slow dissociation from the receptor. The high affinity has made the α-toxin invaluable in the isolation of the receptor. Other toxins such as histrionicotoxin isolated from the skin of a South American frog block the receptor noncompetitively, most likely by altering its channel function. Clearly these toxins bind to distinct sites on the receptor (Taylor et al. 1983; Changeux et al. 1984). Unique among the structures of the receptor antagonists are the diterpenoid coral toxins from the genus *Lophogorgia*. These agents, known as the lophotoxins, are devoid of nitrogen and are uncharged (Fenical et al. 1981). In addition, lophotoxins show apparent irreversibility in their inhibition but, as shall be documented, inhibition results from a true covalent interaction with the receptor. The covalent interaction is of particular value in defining the lophotoxin recognition site, which appears to share characteristics with the agonist site.

ACETYLCHOLINE RECEPTOR STRUCTURE

Nearly two decades of study have shown that the acetylcholine receptor is a pentameric molecule of four subunits α, β, γ, δ, present in the stoichiometry of $\alpha_2\beta\gamma\delta$ (Taylor et al. 1983; Changeux et al. 1984). The subunits show substantial homology with each other and have probably diverged from the same primordial gene. The subunits encircle a channel (Fig. 2); each subunit has an extracellular and intracellular exposure where the peptide backbone spans the membrane multiple times. Only the two α-subunits recognize agonists, and cooperative binding profiles can be detected for agonists. Upon binding of two agonist molecules, a conformational change is induced whereby the channel opens. Presumably, this event occurs by a concerted torsional movement of the subunits. Channel opening is transient ($\sim$1 msec duration) and the associated depolarization is largely a consequence of an inward Na^+ current through the channel.

Our initial studies of lophotoxin action utilized a culture system of cloned muscle cells where occupation of the receptor on the cell surface, and the functional response can be measured simultaneously (Culver et al. 1984, 1985). Occupation of the receptor was ascertained by the ability of lophotoxin to block the initial rate of [^{125}I] α-toxin binding. Block of the functional response entails measuring antagonism of agonist-elicited influx of $^{22}Na^+$ through the receptor channel. The kinetics of lophotoxin inhibition of the receptor are shown in Figure 3. A greater block of the functional response of Na^+ permeability than occupation of the binding site occurs in an equivalent concentration and duration of exposure to lophotoxin. This is consistent with previous data with the snake α-toxins that established that occupation of one of the two agonist sites with α-toxin is sufficient to block the functional response to subsequent agonist challenge (Sine and Taylor 1980; Taylor et al. 1983) (cf. Fig. 4). A more precise analysis of lophotoxin block of the functional response versus its occupation of sites actually shows a curve that lies below the parabola predicted for random occupation of the two sites. This demonstrates that lophotoxin shows a preferential occupation for one of the two α-toxin sites (Fig. 5). The snake α-toxins show equal preference for the respective binding sites on the two α-subunits while the alkaloid antagonists and lophotoxin show a preference for one of the two subunits. Interestingly, experiments utilizing protection of lophotoxin inactivation by the reversible alkaloid antagonists show that the lophotoxin preferred α-subunit is the one of lower affinity for alkaloid antagonists (Culver et al. 1985). The two α-subunits do not differ in primary sequence so the different

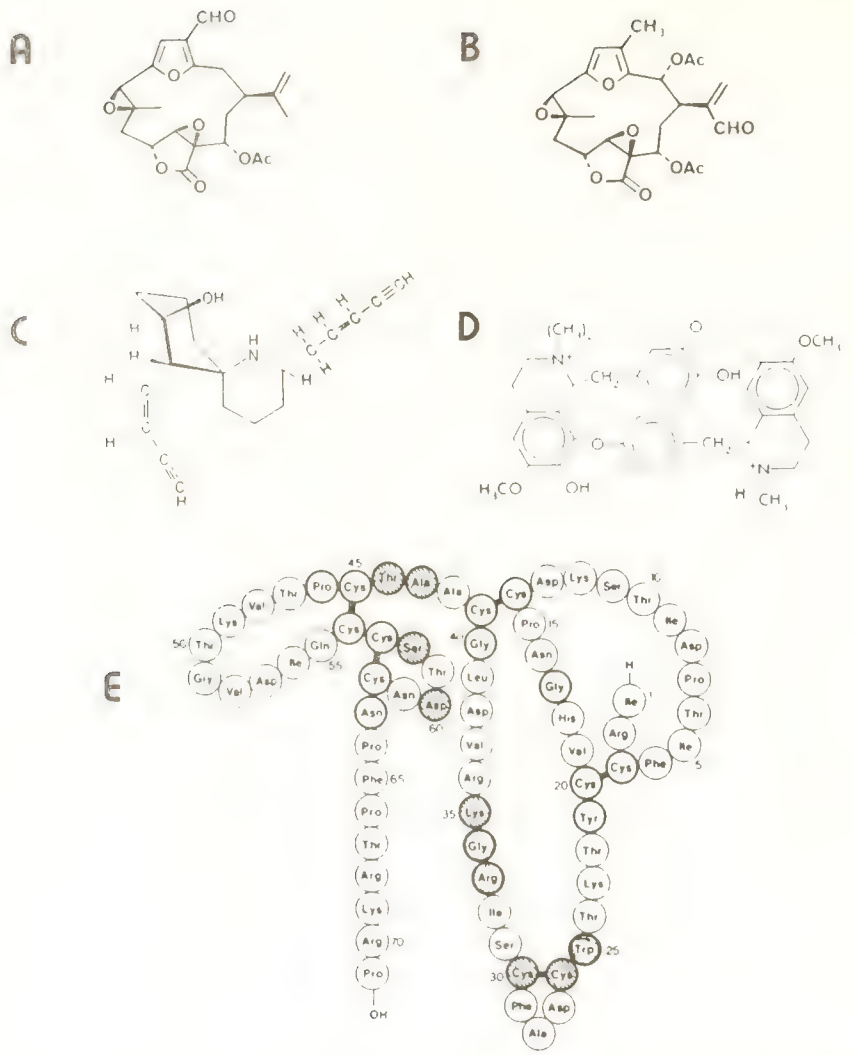
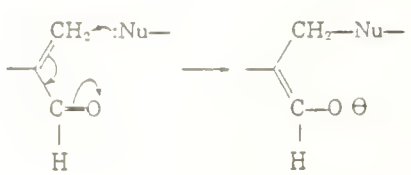


FIGURE 1. Structure of lophotoxin and its active analogue, the alkaloid antagonists, non-competitive inhibitors, and cobra α-toxins. Among a large series of diterpenoid toxins from *Lophogorgia*, lophotoxin (A) and lophotoxin analogue I (B) show the greatest activity. Histrionicotoxin (C) is an example of a non-competitive inhibitor that binds to a separate site. d-Tubocurarine (D) is an example of a plant alkaloid that is a reversible, competitive antagonist. The peptide α-toxins such as the cobra venom α-toxin from *Naja naja siamensus* shown here (E) exhibit competitive but apparent irreversible inhibitor.

ligand specificities of their respective sites arise from either post-translational modification or from the fact that the two α-subunits do not have equivalent subunit neighbors (Taylor et al. 1983). Consistent with the finding that lophotoxin acts at the primary recognition site, we find that agonists and antagonists prevent irreversible inhibition by lophotoxin, while noncompetitive allosteric inhibitors do not protect against lophotoxin inhibition (Fig. 6).

STRUCTURE ACTIVITY CONSIDERATIONS AND MECHANISM OF ACTION

We have also examined a series of lophotoxin analogues for their capacity to inhibit the receptor (Culver et al. 1985). Several were inactive, but within the series, lophotoxin analogue I (LA-I) (Fig. 1) exhibited an activity comparable to lophotoxin. Curiously, we found that LA-I maintained its activity in isolated membrane preparations while lophotoxin itself showed diminished activity in isolated preparations when compared with intact cells. This suggests that lophotoxin may have to be activated within the cell in order to inactivate the receptor irreversibly. Both lophotoxin and LA-I contain an aldehyde spatially removed from an acetoxy group. We have assumed that the acetoxy group in lophotoxin and acetylcholine may occupy the same site. We envision that irreversible action arises either through a Schiff base formation involving the aldehyde, or perhaps a Michael addition. In the case of LA-I, the terminal isopropenyl region shows α, β unsaturation to the carbonyl. Nucleophilic attack could occur by the following mechanism where Nu stands for the nucleophile.



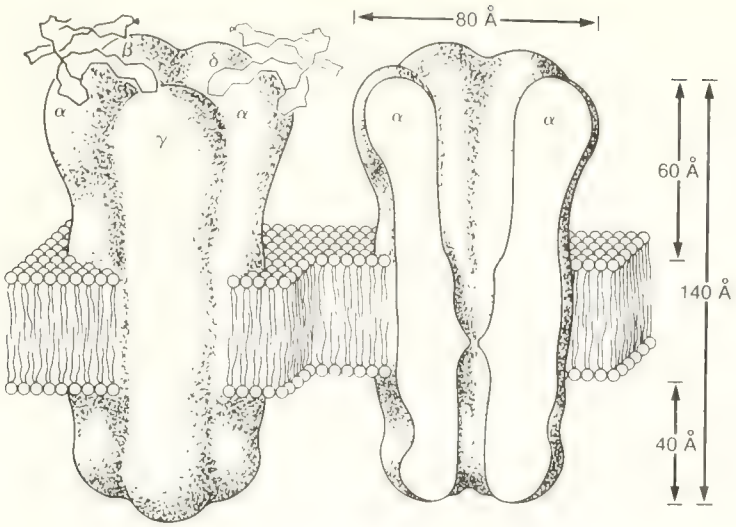
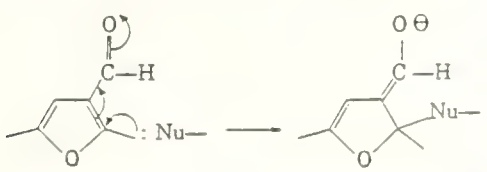


FIGURE 2. Structure of the acetylcholine receptor. Shown are views of the acetylcholine receptor as a pentamer of four distinct subunits of stoichiometry with $\alpha_2\beta\gamma\delta$. The individual subunits of molecular weight of ~55 kD form the outer perimeter of an internal channel and span the membrane. The α-subunits constitute the recognition site for agonists, competitive antagonists, the snake α-toxins, and lophotoxin.

For lophotoxin, a similar mechanism might be envisioned within the furan ring.



In fact, an ethanol adduct that has been isolated during lophotoxin purification provides additional evidence for the reactivity of the furanoaldehyde moiety.

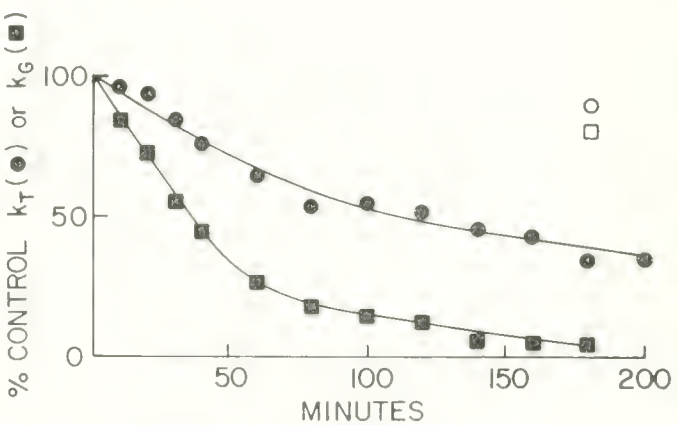


FIGURE 3. Time dependence for lophotoxin inhibition of [^{125}I] α-toxin binding and carbamylcholine-stimulated $^{22}Na^+$ permeability in intact BC3H-1 cells. Cells were exposed to 2 μM lophotoxin for the indicated durations, then subjected to initial rate determinations of [^{125}I] α-toxin binding (●) or 60 μM carbamylcholine-stimulated $^{22}Na^+$ permeability (■) without removal of the lophotoxin. α-Toxin association and $^{22}Na^+$ flux rate constants (κ_T and κ_G, respectively) are expressed as a percentage of control values obtained from cells that were equilibrated in buffer alone. Open symbols denote [^{125}I] α-toxin binding (○) and $^{22}Na^+$ permeability (□) after 3-hr exposure to buffer containing 1% dimethyl sulfoxide (solvent control) (from Culver et al. 1984).

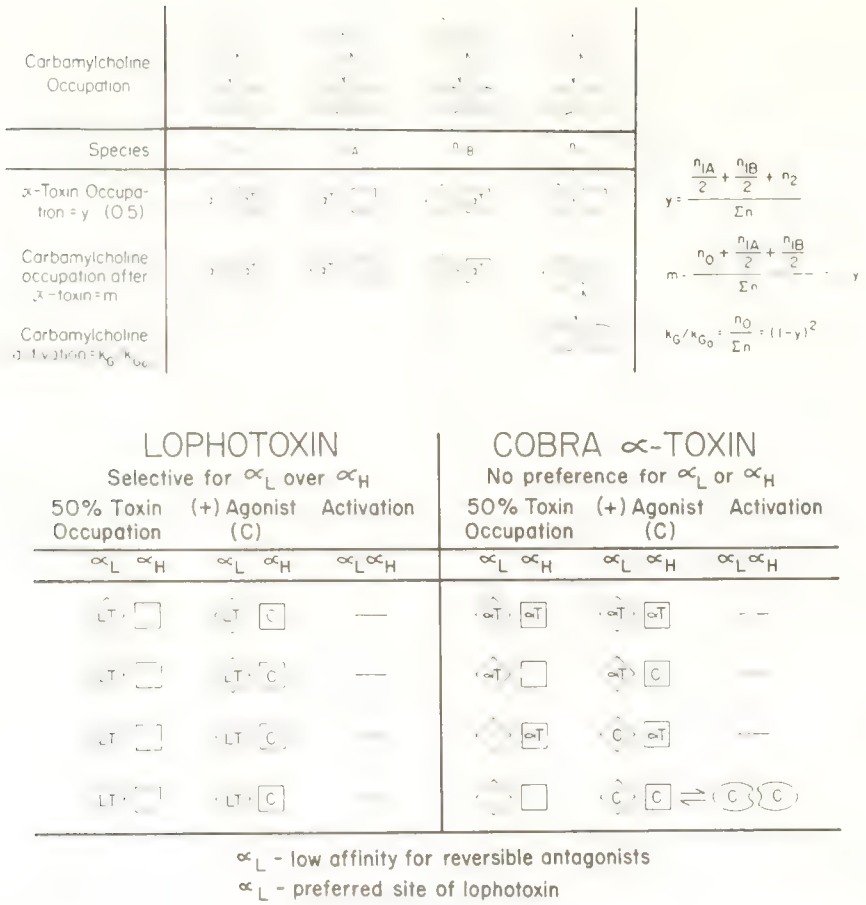


FIGURE 4. Top. Relationship between α-toxin occupation (Y) and the diminution of the permeability response of the receptor (κ_G/κ_{GO}). The receptor behaves as a dimer of functional sites. The model shows α-toxin not distinguishing between the sites on the α-subunits. Hence, toxin occupation will result in a binomial distribution of occupied receptor species. Upon subsequent addition of the agonist, carbamylcholine (C), only the species with both sites unoccupied by α-toxin will respond.

Bottom. Relationship between lophotoxin (LT) and cobra α-toxin (αTX) occupation of the receptor and the block of the functional response. Shown are the two sites on the respective α-subunits. Occupation of both subunits by agonist A is required for channel opening. Cobra α-toxin does not distinguish between α-subunits and thus it will distribute to yield a binomial distribution of receptor occupied species. In this circumstance 50% receptor occupation will yield 75% block of the response. In contrast, lophotoxin exhibits a preference for one of the two α-subunits (α_L). Thus, 50% receptor occupation gives >75% block of the response.

IRREVERSIBILITY OF LOPHOTOXIN ACTION AND RECEPTOR LABELING BY LOPHOTOXIN

The observation that antagonism of receptors cannot be reversed by excessive washing of the preparation does not by itself establish covalent binding. Highly hydrophobic agents may be retained by the preparation or, as in the case of the peptide α-toxin, dissociation of the ligand-receptor complex may be sufficiently slow as to give the appearance of complete irreversibility. To establish that labeling by lophotoxin is covalent, we conducted two types of experiments. First, intact cells were treated with lophotoxin to inactivate the receptor. Membranes were then isolated and the receptors solubilized in 1% Triton X-100. Under these conditions we found that fractional lophotoxin inhibition was the same when we examined the residual sites on the intact cell and on the solubilized receptor (Culver et al. 1985). Thus, the inhibition by lophotoxin survives isolation of the membranes and subsequent solubilization of the membrane associated receptor.

We have also labeled the lophotoxin analogue (LA-I) by sodium borotritide ($NaBT_4$) reduction to the alcohol and subsequent back oxidation to the aldehyde. In this circumstance, the aldehydic hydrogen becomes labeled to a specific activity of 0.1–1.0 Ci/mmole. Membranes prepared from electric organs of the electric ray *Torpedo californica* were incubated with [^{3}H]LA-I, and proteins were separated by electrophoresis in the presence of sodium dodecyl sulfate. A single 40-kilodalton band migrating in the position of the α-subunit was labeled (Fig. 7). In addition, this labeling was prevented by prior incubation of cobra α-toxin. Thus, we have shown that selective lophotoxin conjugation on the α-subunit results in inactivation of function. These observations clearly establish the covalent nature of the association and are consistent with the conclusions on the inactivation experiments in the intact cell.

Covalent labeling of the receptor offers several interesting possibilities. For instance, Karlin (1969) employed maleimidobenzyl-trimethyl ammonium (MBTA) to inactivate the receptor. To achieve irreversible labeling of the receptor with MBTA requires prior reduction of a cystine to a cysteine. This alters the specificity of the receptor, thus labeling does not occur with a completely native receptor. Nevertheless, these pioneering studies have been extended to show that labeling occurs on

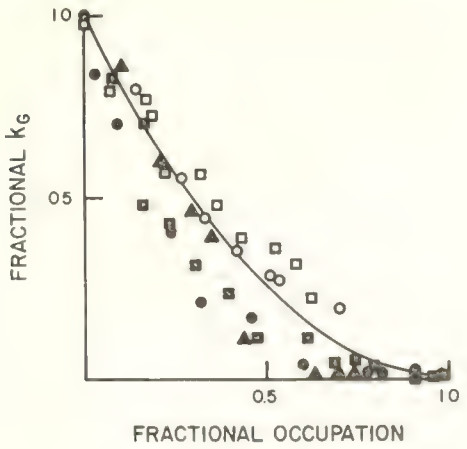


FIGURE 5. Functional capacity of receptors following progressive degrees of irreversible occupancy by lophotoxin or α-toxin. ●, ■, ▲ (three experiments), cells were exposed to lophotoxin (0.06–100 μM) for 120 min, then washed with four 3-ml changes of buffer. Plates were then divided into two groups and assayed in parallel for either 60 μM carbamylcholine-stimulated $^{22}Na^+$ permeability or the initial rate of [^{125}I]-α-toxin binding determinations. Fractional flux (κ_G) values were calculated relative to controls (κ_{Gmax}) which were treated in an identical manner, but with a buffer containing 1% dimethylsulfoxide. Fractional irreversible occupation by lophotoxin (abscissa) was determined from the extent of inhibition of the initial rate of [^{125}I] α-toxin finding relative to controls. ○, □ (two experiments), cells were exposed to unlabeled α-toxin (4.6 nM) for increasing durations up to 100 min and then washed with four 3-ml changes of buffer. Parallel determinations of carbamylcholine-stimulated $^{22}Na^+$ flux and [^{125}I] α-toxin binding were then conducted as described above. For reference, the solid line shows the parabolic relationship between the fractional permeability (κ_G/κ_{Gmax}) and the fraction of sites (y) occupied by a nonselective irreversible inhibitor ($\kappa_G/\kappa_{Gmax} = (1 - y)^2$) (from Culver et al. 1984).

either cysteine 192 or cysteine 193 (Kao et al. 1984). Importantly, this approach has defined part of the binding surface of the receptor. Several quaternary ligands such as p-(N,N-dimethylamino) benzene diazonium (DDF) and p-(trimethylammonium) benzene diazonium (TDF) inactivate the receptor and labeling also occurs on the α-subunit (Weiland et al. 1979; Dennis et al. 1987). Subsequent studies with DDF have shown that tryptophan 184 is the site of labeling. Lophotoxin's unique structure being devoid of a charge, and its chemical reactivity with the native receptor should add another dimension to defining the agonist binding site. Secondly, reduction following lophotoxin labeling with $NaBH_4$ or $NaBH_3$ CN offers an alternate means of examining the chemistry of the reaction.

The evolutionary advantage of toxins that block voluntary

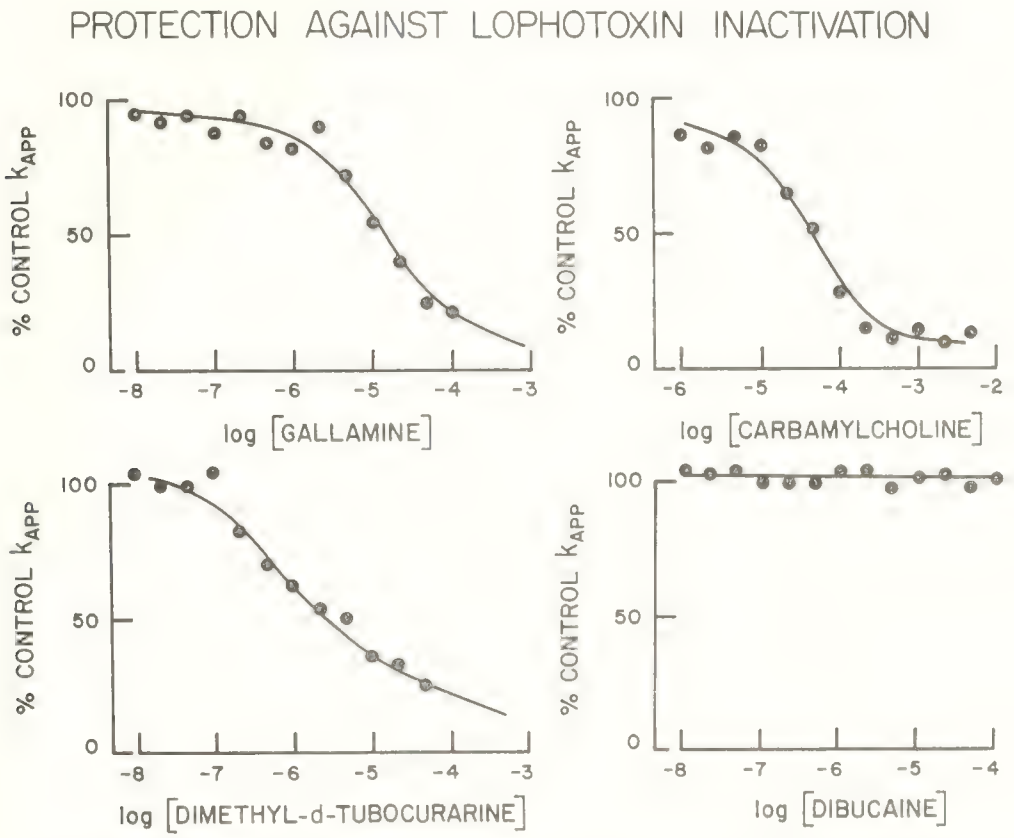


FIGURE 6. Concentration dependence for agonist and antagonist protection against lophotoxin inhibition of [^{125}] α-toxin binding. Cells were first equilibrated for 20–30 min with solutions containing various concentrations of the indicated reversible ligands. Each solution was then replaced with one containing an identical concentration of reversible ligand and 10 μM lophotoxin, and which cells were incubated for 120 min. Cells were washed extensively (6 × 3 ml), then subjected to initial rate determinations of [^{125}I] α-toxin binding. The apparent rate (k_{app}) of lophotoxin inhibition of radiolabeled α-toxin binding was calculated for each treatment. Apparent inhibition rates are expressed on the ordinates as a percentage of that obtained from control plates that were exposed to 10 μM lophotoxin for 120 min in the absence of competing ligand (from Culver et al. 1984).

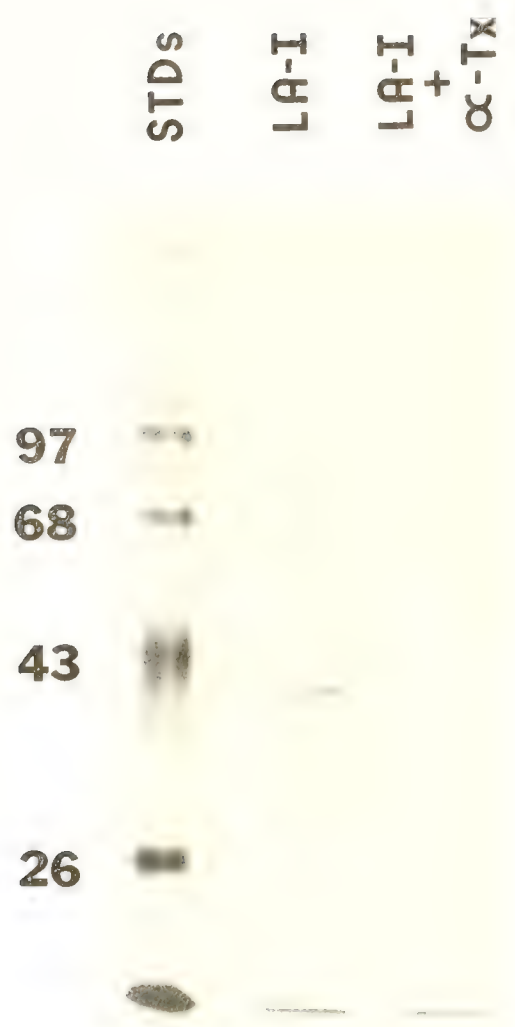


FIGURE 7. Covalent labeling of the acetylcholine receptor from *Torpedo californica* membranes enriched in receptor (specific activity 1.2 nmoles/mg of protein) were incubated with 100 μM [^{3}H] lophotoxin analogue I (LA-I) for 1 hr at room temperature. The receptor was solubilized from the membranes with 1% SDS and run on a 10% polyacrylamide gel in the presence of sodium dodecylsulfate. The gel was soaked in enhancer, dried, placed next to Kodak X-Omat film and exposed for 10 days. Left lane: [^{3}H] molecular weight standards. Middle lane: Receptor incubated with [^{3}H] LA-I. Right lane: Receptor blocked with a stoichiometric excess of α-toxin prior to incubation with [^{3}H] LA-I.

motor activity is clear in terms of both a predator and protective function for the organism. The nicotinic acetylcholine receptor appears to be a preferential site of toxin action within the neuromuscular junction. What is most intriguing is the great structural and mechanistic diversity of toxins that converge to achieve a similar antagonism of a specific physiologic function.

ACKNOWLEDGMENTS

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Olfactory Receptors of Crustaceans with Similarities to Internal Receptors for Neuroactive Substances

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INTRODUCTION

The chemical senses are well developed in many marine organisms and function to provide detailed information about the chemical composition of the external aquatic environment. Indeed, a broad spectrum of adaptive behavioral responses are triggered by specific chemical signals that are detected externally. These responses include food recognition and ingestion (Carr 1982; Mackie 1982), deterrence of feeding (Faulkner and Ghiselin 1983), predator avoidance (Mackie 1970), mate recognition (Gleeson 1980), gamete release (Müller et al. 1979), selection of a substratum for settlement and metamorphosis by larvae (Morse and Morse 1984), homing by migratory species (Stabell 1984), social interactions between conspecifics (Liley 1982), and maintenance of symbiotic relationships (Ache 1974). General reviews of chemically stimulated behavior in marine animals are provided by Mackie and Grant (1974), Gleeson (1978), Daloze et al. (1980), Atema (1985), and Carr (1988).

In this paper we show that many of the external chemical agents that evoke specific behavioral responses in marine animals are substances with neuroactive functions in internal tissues. We will focus on three recently studied examples of such chemical agents: the adenine nucleotides AMP and ATP, and the sulfur-containing amino acid taurine. A description is given of the properties of the external chemoreceptors in marine crustaceans that are selectively activated by these compounds. Similarities are shown to exist between these chemoreceptors and the internal receptors that are activated by these substances. Further, it is shown that certain perireceptor events (e.g., metabolic processing and/or uptake of these compounds) are similar in both internal tissues and in a chemosensory organ. The presentation concludes with some observations on the suitability of the olfactory system of the lobster as a model to study postsynaptic processes that mediate and regulate the activities of several neuroactive agents.

SIMILARITIES BETWEEN EXTERNAL CHEMORECEPTORS AND RECEPTORS FOR INTERNAL NEUROACTIVE AGENTS

Chemoreceptors that monitor specific chemicals appearing in the external aquatic environment play a role that is functionally quite similar to that of synaptic receptors that monitor specific chemicals appearing in a synaptic cleft. With both receptor types, information is obtained about signal molecules appearing in an aquatic milieu external to the receptor cell itself. Both receptor types include reversible binding sites on a cell membrane; in both cases the occupancy of these sites is coupled to a transduction mechanism that induces a change in the excitatory state of the membrane. Further, as will be described later, both receptor types may be influenced by the interactions of excitant molecules with enzyme or transport processes that reduce the concentration of signal molecules in the receptor environment (see also Getchell et al. 1984).

Beginning with Haldane (1954), the similarities between external and internal "chemosensory" systems have prompted biologists to consider the hypothesis that many receptors in internal tissues trace their origins to certain external chemoreceptors of primitive aquatic organisms (Lentz 1968; Kittredge et al. 1974; Lenhoff and Heagy 1977). This hypothesis is supported by the fact that, as shown in Table 1, at least 14 sub-

TABLE 1. NEUROACTIVE SUBSTANCES THAT ACTIVATE BOTH EXTERNAL CHEMORECEPTORS AND RECEPTORS IN INTERNAL TISSUES.

Neuroactive agent	Organism with chemoreceptors for agent	Behavior affected	Reference*
Acetylcholine	Dinoflagellate	Light sensitivity	1
	Ciliate protozoan	Swimming	2
	Human oocyte	Unknown	3
Adenosine	Army worm	Feeding	4
AMP	Shrimp; octopus	Attraction	5, 6
	Mosquito larva	Attraction	7
	Spiny lobster	Unknown	8
ATP	Assassin bug	Liquid ingestion	9
	Mosquito	Liquid ingestion	10
	Spiny lobster	Attraction	11
Cyclic AMP	Slime mold	Aggregation	12
DOPA†	Oyster larva	Settlement and metamorphosis	13
α- and β-Ecdysone	Spiny lobster	Unknown	14
Epinephrine	Ciliate protozoan	Glucose uptake	15
GABA†	Abalone larva	Settlement and metamorphosis	16
	Human oocyte	Unknown	17
Glutamate	Colonial alga	Induces sexual stage	18
	Spiny lobster	Unknown	19
	American lobster	Unknown	20
Glycine	Mud snail	Proboscis extension	21
	Spiny lobster	Feeding	11
PGA2†	Fish	Feeding inhibition	22
Taurine	Spiny lobster	Attraction	23
	American lobster	Unknown	24
Thyroxine	Ciliate protozoan	Phagocytosis	25

* Reference numbers: 1—Forward 1977. 2—Doughty 1978. 3—Eusebi et al. 1984. 4—Ma 1977. 5—Carr and Thompson 1983. 6—Chase and Wells 1986. 7—Barber et al. 1982. 8—Derby et al. 1984. 9—Friend and Smith 1982. 10—Galun et al. 1985. 11—Zimmer-Faust 1987. 12—Mato et al. 1978. 13—Coon et al. 1985. 14—Spencer and Case 1984. 15—Csaba 1980. 16—Morse et al. 1979; Trapido-Rosenthal and Morse 1986. 17—Dolci et al. 1985. 18—Starr et al. 1980; Maier and Muller 1986. 19—Derby and Ache 1984. 20—Derby and Atema 1982; Borroni et al. 1986. 21—Carr 1967. 22—Gerhart 1984. 23—Johnson and Ache 1978. 24—Johnson and Atema 1983; Borroni et al. 1986. 25—Csaba et al. 1977.

† DOPA = dihydroxyphenylalanine; GABA = γ-aminobutyric acid; PGA2 = prostaglandin A_2.

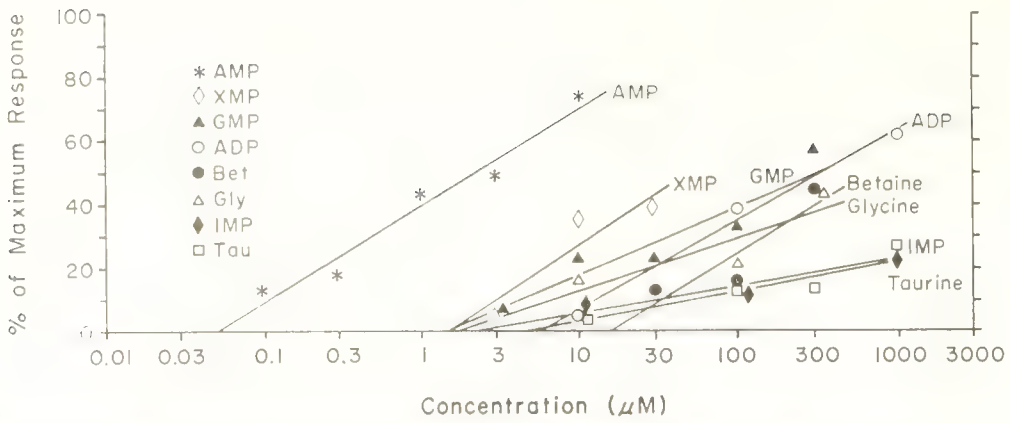

FIGURE 1. Behavioral dose-response functions for excitatory substances tested on the shrimp *Palaemonetes pugio*. Modified from Carr and Derby (1986).

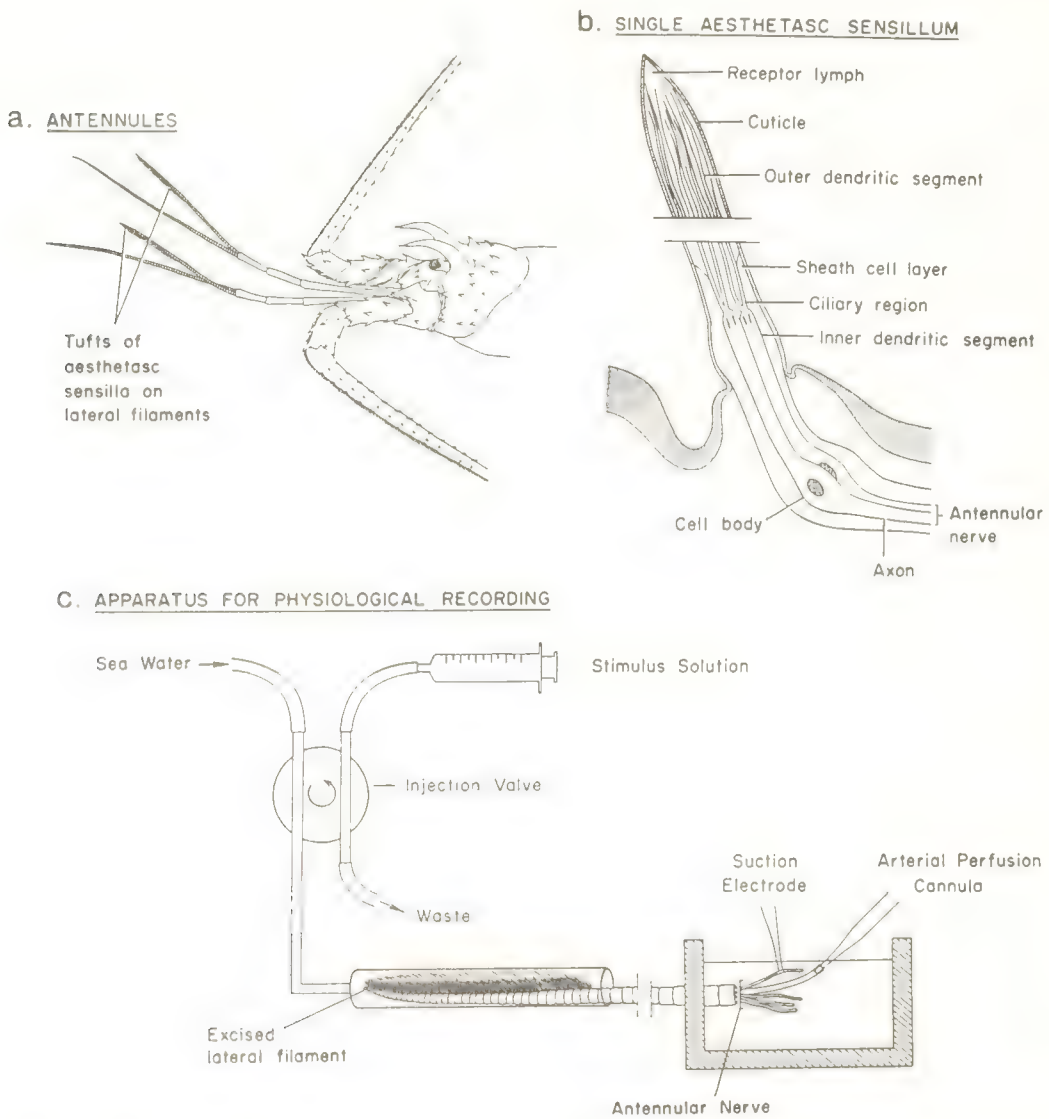

FIGURE 2. (a) Head of the spiny lobster showing lateral filaments of the antennules with rows of aesthetasc sensilla. (b) Schematic of an aesthetasc showing dendritic terminals within the cuticular sheath. Some details of structure from Grünert and Ache (1988). (c) Diagram of the stimulus delivery and recording apparatus employed to analyze chemoreceptor cells in the lobster antennule. Further details are given in text.

stances are known to activate receptors present in both internal tissues and on the membranes of external chemosensory cells; these substances include the following: acetylcholine, adenine nucleotides, DOPA, ecdysones, epinephrine, GABA, glutamate, prostaglandin A_2, taurine, and thyroxine. It may be particularly revealing that the surface membrane of an unfertilized mammalian oocyte possesses receptors for the transmitters acetylcholine and GABA (Eusebi et al. 1984; Dolci et al. 1985). Since a mammalian oocyte serves as the "progenitor" of a complex multicellular organism, it is interesting that its initial receptors are exposed to chemical signals appearing in an environment external to the oocyte itself. Following fertilization, developmental events distribute the daughter cells, together with their heretofore external receptors, into the various internal layers of the embryo. These findings with oocytes provide an ontogenetic argument supporting the hypothesis that receptors monitoring chemical signals in an organism's internal milieu may have evolved from receptors that originally monitored these same chemical signals in an external environment (Haldane 1954; Lentz 1968). Although such an hypothesis is difficult to prove or disprove in its entirety, certain marine organisms do have external chemosensory receptors, plus mechanisms for the inactivation of excitants, that are remarkably similar to internal systems that recognize and inactivate many of the very same neuroactive agents. As will be shown, these similarities indicate that the chemosensory systems of certain marine organisms have the potential for being convenient models in studies of recognition systems for several neuroactive agents (see also Lenhoff and Heagy 1977).

BEHAVIORAL CHEMOATTRACTANTS OF A SHRIMP

The marine shrimp *Palaemonetes pugio* is an opportunistic scavenger that normally feeds upon detritus and small invertebrates (Welsh 1975; Bell and Coull 1978). In the laboratory, *P. pugio* is quickly attracted to, and feeds upon, the soft tissues of many organisms. Chemoattractants for this shrimp have been identified using a bioassay procedure involving the delivery of dissolved chemicals to groups of animals in compartmented boxes (Carr and Derby 1986). Each positive response consists of a shrimp coming to and grasping the delivery device. For each attractant, potency is determined by recording the number of positive responses over a range of concentrations.

The low molecular weight components of aqueous extracts prepared from crab, shrimp, oyster, and mullet are highly attractive to *P. pugio* (Carr et al. 1984; Carr and Derby 1986). Analyses of these components in each extract resulted in the identification of 27 amino acids, 3 quaternary amines, 3 organic acids, and 8 nucleotides and related purines. When bioassayed individually, only 8 of the 41 substances served as attractants to the shrimp. These attractants are the nucleotides AMP, XMP, GMP, ADP, and IMP, the amino acids glycine and taurine, and the quaternary amine betaine (see Fig. 1). Except for betaine, all of these chemoattractants are known either for their neuroactive effects in internal tissues, or, as in the case of the nucleotides XMP, GMP and IMP, occur within the response spectrum of known internal receptor types.

The purine nucleotide adenosine 5′-monophosphate (AMP) is clearly the single most potent chemoattractant identified (Fig. 1). Moreover, bioassays of AMP analogs and a purinergic receptor antagonist, theophylline, suggested that the behavioral response of the shrimp to AMP was mediated by chemoreceptors akin to the P_1-type purinoceptors found in the internal tissues of mammals (Carr and Thompson 1983).

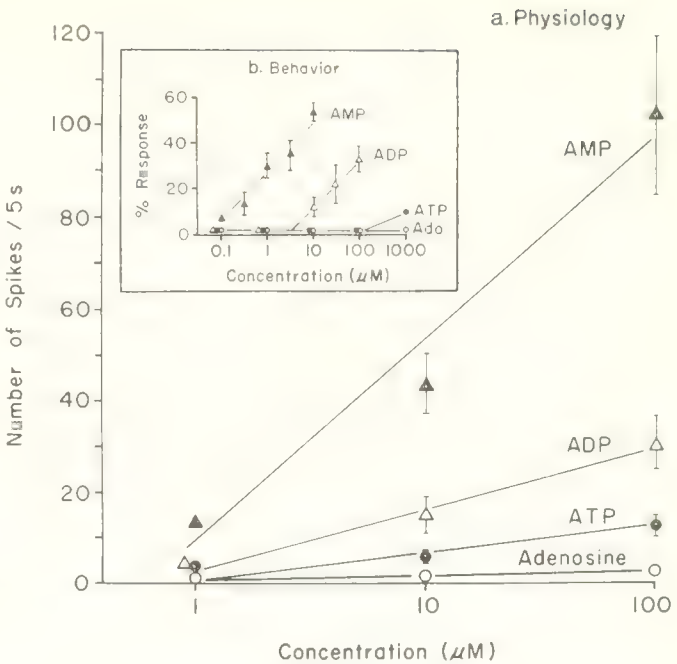

FIGURE 3. (a) Physiological dose-response functions for adenine nucleotides and adenosine in olfactory neurons of the spiny lobster, *Panulirus argus*. (b) (Inset) Behavioral responses to the same substances tested in the shrimp *Palaemonetes pugio*. Data points are mean values ± SEM. From Derby et al. (1984).

PURINERGIC CHEMORECEPTORS OF THE SPINY LOBSTER

Physiological evidence that purinergic chemoreceptors exist in the olfactory organ of a marine crustacean was obtained in studies with the Florida spiny lobster, *Panulirus argus*. This animal, a much larger crustacean than the shrimp *P. pugio*, had been shown in earlier studies to be ideally suited for electrophysiological studies of olfaction (Ache 1982). The olfactory organ of the spiny lobster consists of dense rows of sensilla (aesthetascs) residing on the lateral filament of each antennule (Fig. 2a). A lateral filament has about 2,000 aesthetascs, each containing the ciliated dendritic terminals of an estimated 320 sensory neurons; somata and axons of these olfactory cells are situated within the lumen of the antennule (Laverack and Ardill 1965; Grünert and Ache 1988). Axons of the olfactory neurons join to form the antennular nerve (Fig. 2b), which projects to the brain.

Physiological responses of olfactory cells in the spiny lobster were obtained from an excised antennular preparation as described by Derby and Ache (1984) and Gleeson and Ache (1985). Briefly, this procedure employs a perfused lateral antennular filament that is inserted into an olfactometer and continuously flushed with artificial sea water (ASW) (Fig. 2c). Chemical stimulation is accomplished by injecting solutions into the carrier flow of ASW passing over the chemosensory sensilla. Extracellular recording of action potentials from single cells is achieved by splitting fascicles from the antennular nerve and applying a

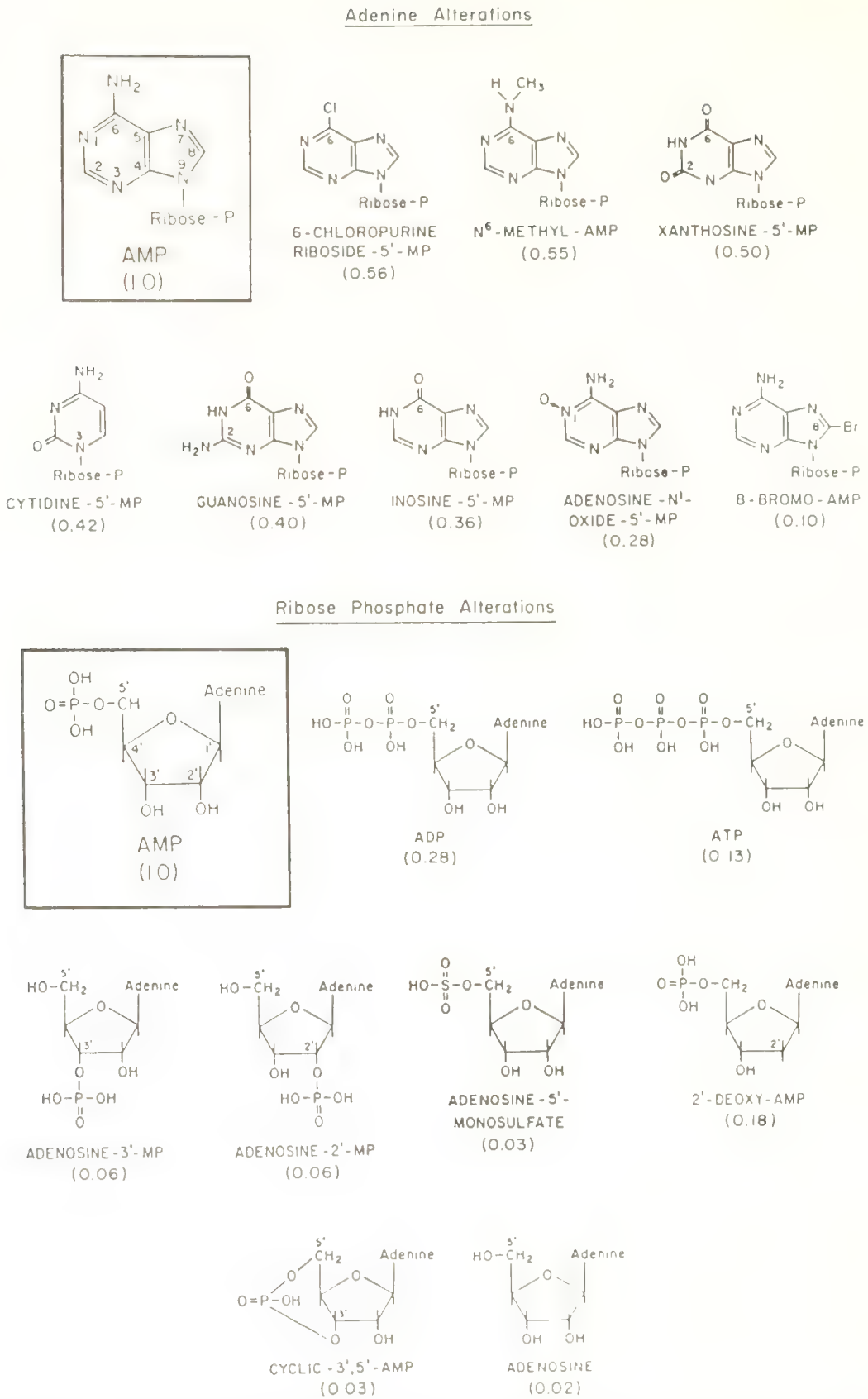

FIGURE 4 Molecular formulae of AMP and analogs tested on AMP-sensitive cells in the antennule of the spiny lobster. Analogs are altered in the adenine moiety (above) or the ribose phosphate moiety (below). The index of relative activity given beneath each substance is the ratio of the intensity of the physiological response to 10 μM of that substance relative to 10 μM AMP. Data from Derby et al. (1984).

suction electrode *en passant* to the exposed axons. Single-unit status of each recording is verified by passing the analog signal through an amplitude discriminator and establishing that action potentials have a common waveform.

Utilizing the procedure summarized above, it was shown that the spiny lobster has nucleotide-sensitive olfactory receptors exhibiting a potency sequence of AMP > ADP > ATP or adenosine (Derby et al. 1984); this potency sequence, measured physiologically, is the same as that measured behaviorally in the shrimp (Fig. 3). Studies of structure-activity relationships (SAR) of AMP and analogs revealed that AMP is the most potent stimulant, and all changes in its structure result in significant decreases in activity (Fig. 4). However, changes in the ribose phosphate moiety result in greater decreases in activity than changes in the purine moiety; note in Figure 4 that seven of the eight analogs with relative activities of less than 0.20 are substances modified in the ribose phosphate region. An additional finding was that the response of the "AMP-best" olfactory cells is antagonized by theophylline (Derby et al. 1984).

In mammals, receptors for purine nucleotides (purinergic receptors) are present on the cell membranes of many types of internal tissues including visceral smooth muscle (Brown and Burnstock 1981), cardiovascular tissue (Olsson et al. 1979), and neurons in both the peripheral and central nervous systems (Burnstock 1980; Phillis and Wu 1981). Burnstock (1978) recognized that purinergic receptors are not homogeneous, and introduced the terms P_1 and P_2 to describe two separate types. Among the distinctive features of the P_1-type (or R-type) receptor are: (1) a potency sequence of adenosine ≥ AMP > ADP ≥ ATP; (2) less tolerance to structural changes in the ribose moiety than in the purine moiety; and (3) antagonism by theophylline and other methyl xanthines (Londos and Wolff 1977; Burnstock 1978; Londos et al. 1980). Hence, the only observed difference between the P_1-type purinoceptor and the "AMP-best" olfactory receptor of the spiny lobster concerns the activity of the non-phosphorylated nucleoside adenosine. Adenosine can activate the P_1-type receptor in internal tissues, whereas a 5′-phosphate group on the adenosine moiety is required to activate the P_1-like chemoreceptor.

The spiny lobster also has a population of "ATP-best" olfactory receptors that are clearly distinct from the P_1-like chemoreceptors (Carr et al. 1986). This second population of chemosensory cells has the following similarities to the P_2-type purinoceptors described by Burnstock (1978): (1) a potency sequence of ATP > ADP > AMP or adenosine (Fig. 5a); (2) broad sensitivity to nucleotide triphosphates including those with changes in both the ribose and adenine moieties; (3) rapid desensitization; and (4) activation by certain slowly degradable analogs of ATP, e.g., β, γ imido ATP, β, γ methylene ATP, and α, β methylene ATP (see also Burnstock and Kennedy 1985).

The P_2-like olfactory cells of the lobster have additional response properties that clearly differentiate them from the P_1-like cells. P_2-like cells give responses to ATP that are of much shorter duration and have fewer impulses per response than characterizes the responses of the P_1-like cells to AMP (Fig. 5b, c, e, f).

P_2-like chemoreceptors with physiological responses very similar to those described above have been identified in a second species, the Pacific spiny lobster, *P. interruptus* (see Zimmer-Faust et al. 1988). Interestingly, in the Pacific species, ATP is also known to function as a potent behavioral stimulant (Zimmer-Faust 1987).

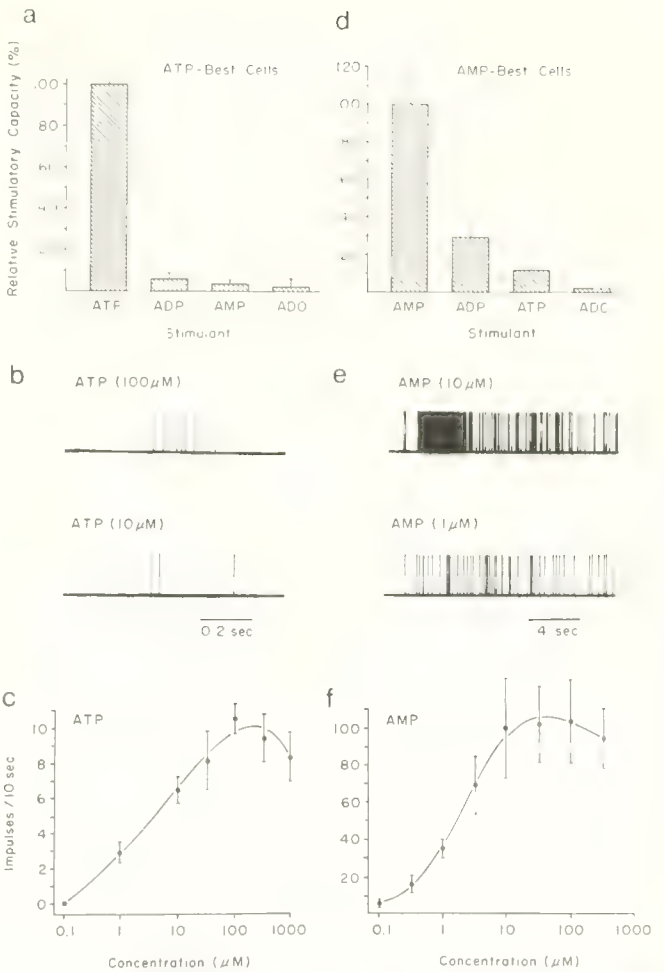

FIGURE 5. Comparisons of physiological response characteristics of ATP-sensitive cells and AMP-sensitive cells in the antennule of the spiny lobster. (a, d) Intensity of the response of each cell type to 100 μM ATP, ADP, AMP and adenosine. (b, e) Response profiles of the two cell types each tested at two concentrations. Note differences in time scale, plus differences in duration and intensity of responses. (c, f) Dose-response functions for populations of each cell type. ATP-sensitive cells gave maximum discharges of about 10.5 impulses/response; AMP-sensitive cells gave maximum discharges of about 104 impulses/response. Data points are means ± SEM. From Carr et al. (1986).

BIOCHEMICAL SYSTEMS FOR THE INACTIVATION OF NUCLEOTIDES AND THE UPTAKE OF ADENOSINE

Examination of the biochemical fate of excitatory nucleotides reveals that additional major parallels exist between the purinergic systems in the olfactory organ of the lobster and the internal tissues of mammals. In mammals, extracellular adenine nucleotides are inactivated by a two-step process involving first, dephosphorylation to yield the nucleoside adenosine; and second, internalization (salvage) of adenosine by an uptake system (Burnstock 1975, 1980). The dephosphorylation step is catalyzed by enzymes termed ectonucleotidases that are present on the external surfaces of neurons, glia, and other types of cell (Kreutzberg et al. 1978; Cusack et al. 1983; Pearson 1985).

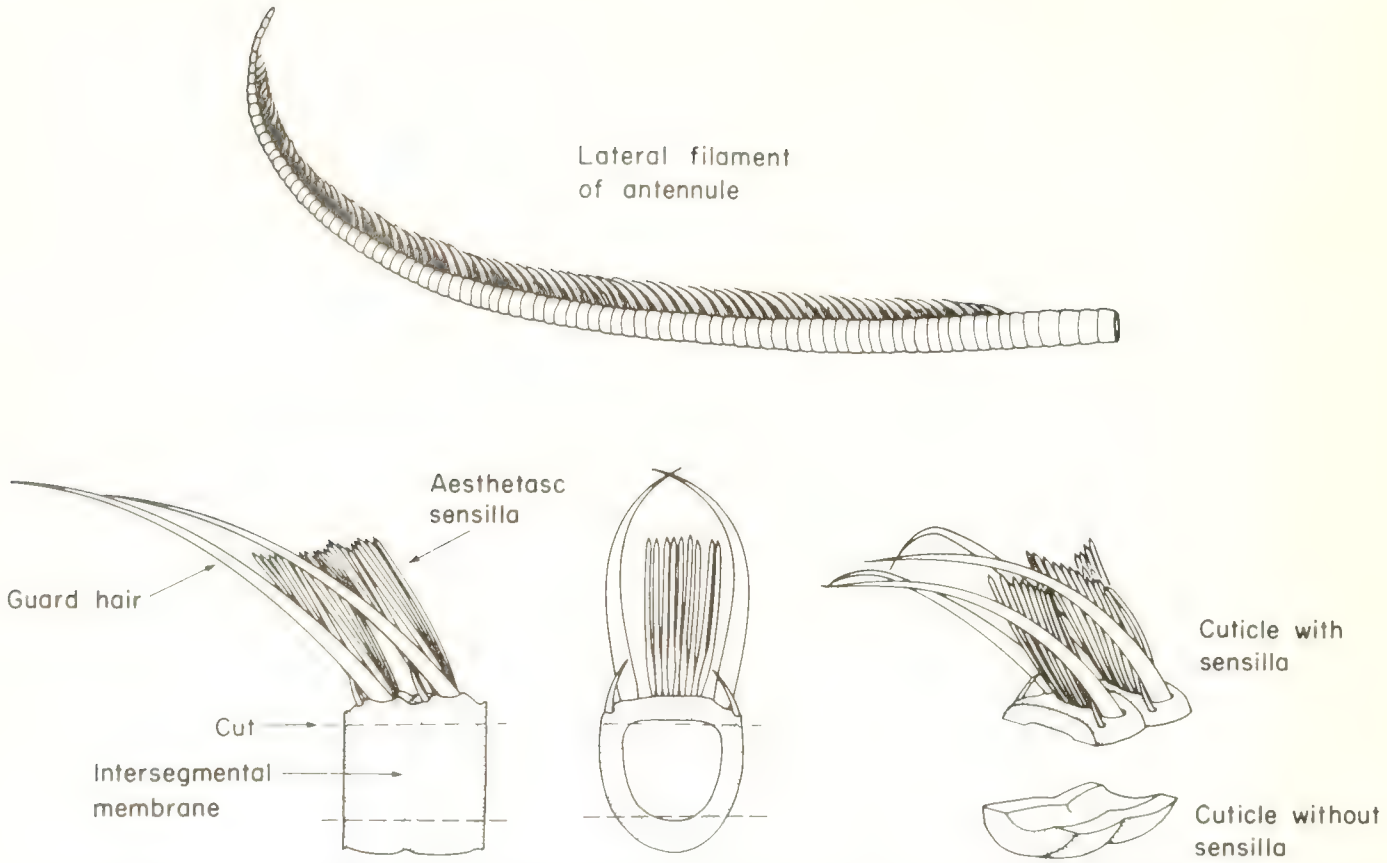

FIGURE 6. Procedure for obtaining sensilla-bearing sections of cuticle from the antennule of the spiny lobster. Lateral antennular filaments are divided into pairs of segments and cut tangentially to yield cuticular sections with attached sensilla. Further details are given in text.

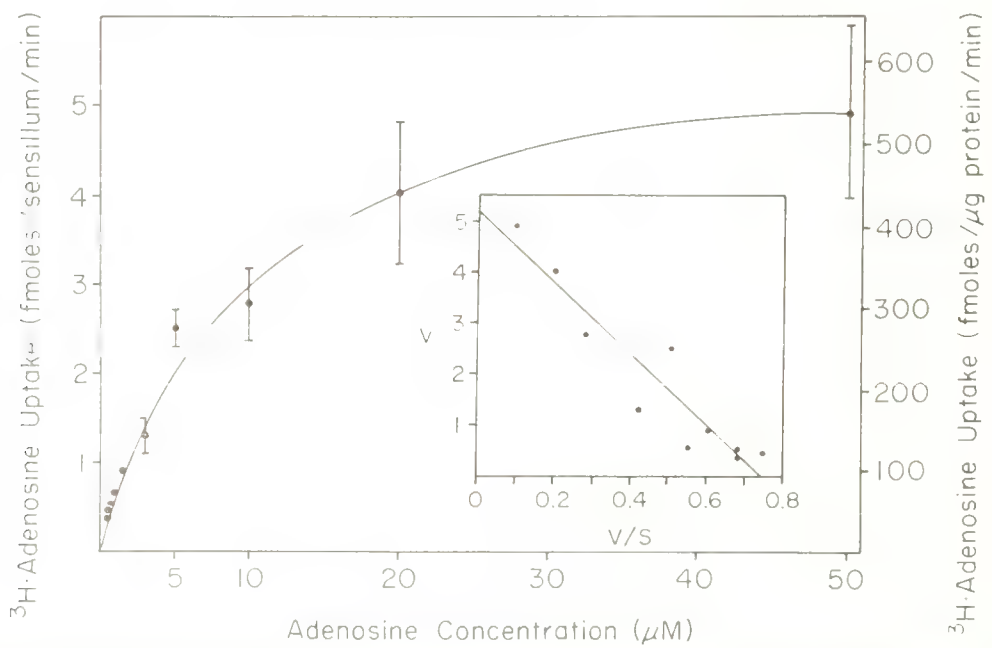

FIGURE 7. Effect of adenosine concentration on adenosine uptake of aesthetasc sensilla of the spiny lobster. Each point is the mean ± SEM of multiple incubations. (inset) Eadie-Hofstee transformation of the above data. The kinetic parameters of adenosine uptake computed from this transformation are K_M = 7.1 μM and V_{Max} = 184 pmoles/mg protein/minute (5.2 fmoles/sensillum/minute). From Trapido-Rosenthal et al. 1987*a*

Internalization of adenosine is accomplished by a specific uptake system thought to involve facilitated diffusion (Hertz 1978; Bender et al. 1981; Kreutzberg et al. 1983).

Studies of the biochemical fate of nucleotides in olfactory sensilla of the lobster are performed using sensilla-bearing sections of cuticle prepared as depicted in Figure 6. Following incubation with radiolabelled compounds, sensilla are collected by filtration, rinsed, digested in NaOH, and the amount of radioactivity determined by liquid scintillation spectrophotometry (Trapido-Rosenthal et al. 1987*a*; Trapido-Rosenthal et al. 1987*b*).

Sensilla incubated with [^{3}H]adenosine in the presence and absence of an excess of unlabelled adenosine exhibit an uptake system for adenosine that is saturable with increasing concentration, shows sodium dependence, and has a K_M of 7.1 μM and a V_{Max} of 5.2 fmoles/sensillum/minute (Fig. 7). The sensilla internalize the adenosine moiety of AMP as rapidly as adenosine itself (Fig. 8a). Evidence that the sensilla contain an enzymatic activity that rapidly dephosphorylates AMP extracellularly and then internalizes the resultant adenosine is revealed in a double-label experiment in which [^{3}H] from adenine-labeled AMP is rapidly internalized whereas [^{32}P] from phosphate-labeled AMP is not (Fig. 8b).

The sensillar mechanisms for the dephosphorylation of an excitatory nucleotide and the uptake of adenosine are quite similar to the mechanisms described earlier for nucleotide inactivation and salvage that occur in internal tissues (Trapido-Rosenthal et al. 1987*a*). In the olfactory sensilla, the dephosphorylation of nucleotides serves as an inactivation step because the product of the dephosphorylation, adenosine, is not an excitant of either type of purinergic receptor identified in the lobster's olfactory organ (Fig. 5a, d).

PHYSIOLOGY AND BIOCHEMISTRY OF THE SENSILLAR TAURINGERGIC SYSTEM

Taurine (2-aminoethanesulfonic acid) is another potent olfactory excitant of the spiny lobster *P. argus*. This ubiquitous amino acid occurs in high concentrations in the tissues of many invertebrates eaten by the lobster and presumably functions as a feeding stimulant (Johnson and Ache 1978). In mammals, taurine plays a role in heart function (Grosso and Bressler 1976), retinal activity (Pasantes-Morales et al. 1972), and neural development (Gaull and Rassin 1979). An hypothesis that taurine may function as an inhibitory neurotransmitter in mammals (Davison and Kaczmarek 1971; Mandel et al. 1976) remains largely untested because a suitable model for the development of selective antagonists is lacking (Barbeau 1982).

Olfactory chemoreceptors selectively activated by taurine and close analogs occur in the antennules of the spiny lobster (Fuzessery et al. 1978; Gleeson et al. 1987). Major similarities exist between the specificity of these olfactory receptors and the taurine recognition systems in mammals. For example, aside from taurine itself, β-alanine and hypotaurine were the most potent analogs tested on taurine-sensitive cells of the lobster, and are also potent competitors for taurine binding sites in rat brain synaptosomes (Hruska et al. 1978; Segawa et al. 1982) and taurine uptake sites in heart (Schaffer et al. 1982). Likewise, uptake of taurine by human blood platelets is inhibited by β-alanine and hypotaurine, but not by 2-aminoethylphosphonic acid (AEP) (Gant and Nauss 1976); AEP is also non-stimulatory to the taurine-sensitive cells of the spiny lobster.

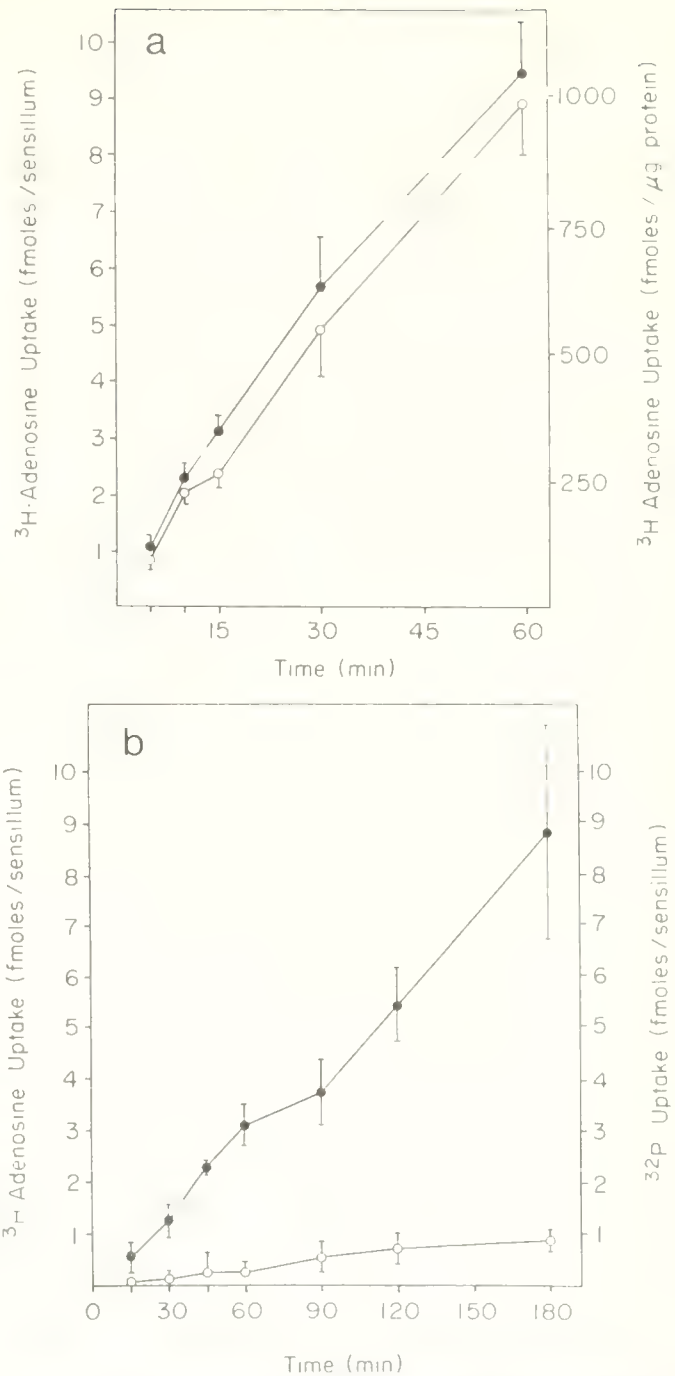

FIGURE 8. (a) Uptake of tritium from equimolar [^{3}H]-AMP (●) and [^{3}H]adenosine (○) by aesthetasc sensilla of the spiny lobster. (b) Double-label determinations of sensillar uptake of [^{3}H] (●) and [^{32}P] (○) from labeled AMP. Tritium labeling was at positions 2 and 8 of the adenine ring; [^{32}P] labeling was at the α-phosphate group. Each data point is the mean ± SEM of multiple incubations. From Trapido-Rosenthal et al. 1987*a*

In addition to having chemoreceptor sites activated by taurine, the olfactory sensilla of the spiny lobster have a taurine uptake system of high specificity that functions to remove this

excitant from the receptor environment (Gleeson et al. 1987). Measurements of the kinetics, specificity, and sodium-dependence of taurine uptake, plus the competitive inhibition of the uptake process by guanidinoethane sulfonic acid (e.g., see Huxtable et al. 1979), indicate that the sensillar uptake system in the lobster has major similarities to that for taurine uptake in mammalian tissues.

OLFACTORY SYSTEM OF THE SPINY LOBSTER AS A MODEL FOR BIOMEDICAL STUDIES OF POSTSYNAPTIC NEURONAL EVENTS

In internal tissues, dendrites of postsynaptic neurons have specific receptors that are activated by neurotransmitters released from presynaptic neurons. Similarly, in the olfactory sensilla of the lobster, dendritic processes of primary chemosensory neurons have receptors selectively activated by several of these same "neuroactive" substances when they occur in the lobster's external environment (Fig. 2b). Chemical stimulation of the dendritic receptors in the sensilla gives rise to a graded receptor potential which in turn results in the generation of action potentials in the axon (Anderson and Ache 1985). Since this process of chemical stimulation is analogous to neurotransmitter activation of a postsynaptic neuron, the olfactory neuron of the lobster represents a potential postsynaptic model system.

The anatomy of the olfactory neurons, coupled with the response specificity of discrete cell populations to particular neuroactive substances, endows this system with several desirable features for use as a model in studies of postsynaptic neuronal events. These features include:

1. The electrical response of a single neuron following exposure to an agonist provides a convenient measure of ligand-receptor interactions.

2. Because these neurons are primary sensory cells, electrical responses are not confounded by synaptic inputs from other neurons.

3. The dendritic processes of the neurons are directly accessible; the composition of solutions reaching the receptor environment can be precisely controlled without altering the medium bathing the remainder of the cell.

4. Biochemical analyses of processes such as reversible receptor binding, and mechanisms for inactivating or clearing excitants from the receptor environment, can be performed in a preparation that is free of components derived from presynaptic neurons.

5. Complementary physiological and biochemical studies can be conducted on the same system.

In addition to the populations of olfactory neurons exhibiting selective sensitivity to AMP, ATP or taurine, other olfactory cells in spiny lobsters have been identified that are activated by glutamate, glycine, ADP, and certain ecdysones (see Table 1 and Carr et al. 1987). Having external chemoreceptor cells that respond to neuroactive substances is not unique to the spiny lobster model. What *is* unique, however, is the array of opportunities that this model affords for working at both the physiological and biochemical levels with a spectrum of selectively sensitive receptor types.

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Importance of Marine Natural Products in the Study of Inflammation and Calcium Channels

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INTRODUCTION

Plant natural products have been an important source of compounds that have assumed importance as research tools and medicines (Gilman et al. 1985). The sea is emerging as a potential source of novel chemical structures that serve as lead compounds and may have medicinal value. The focus of this report will be on marine natural products that may have anti-inflammatory activity or have helped our understanding of the mechanisms of inflammation and the role that Ca^{2+} plays as a second messenger in this process. The results of a recent survey of the literature are shown in Table 1. Toxins from marine organisms have been the focus of most scientific investigations because of their dramatic harmful effects (pain, paralysis, death, etc.). These toxins have often been useful as research tools. One of the best known examples is tetrodotoxin, a sodium channel inhibitor (Catterall 1980). However, this type of compound represents only a small fraction of the diversity available from the sea.

In addition to being a source of compounds, marine organisms have been important as model systems and in shaping our thinking of inflammation and immune responses. A detailed discussion is beyond the scope of this report. However, a few examples illustrate the point. Dunham et al. (1985) reported that the Ca^{2+}-dependent aggregation of sponge cells resembles the stimulus–response coupling of neutrophils and platelets. Dissociated sponge cells in Mg–Ca^{2+}-free seawater with 2.5 mM EDTA aggregate upon exposure to Ca^{2+} (>5 mM) and Ca^{2+} ionophore. Inflammatory agents such as LTB_4 or urushiols provoke aggregation, while non-steroidal anti-inflammatory drugs inhibit aggregation. Based on these findings, the use of sponge cells to identify new anti-inflammatory compounds has been patented (Dunham and Weissman 1986).

Scofield et al. (1982) showed that in colonial tunicates, self–nonself discrimination may represent the evolutionary precursor(s) to major histocompatibility complex genes (MHC). Prendergast et al. (1983) reported that sea star factor and rabbit lymphokines show cross phylum activity suggesting a phylogenetic link of lymphokines.

IMPORTANCE OF PHOSPHOLIPASES AND CALCIUM IN INFLAMMATION

Pro-inflammatory stimuli, as well as a wide variety of hormones, neurotransmitters, and growth factors, bind to receptors and activate an intracellular signal(s), producing their characteristic effects on cell function in part through mobilization of Ca^{2+} (Berridge and Irvine 1984; Sekar and Hokin 1986). Cells can mobilize calcium from intracellular and extracellular sources. A key step in the action of Ca^{2+} mobilizing agonists that release Ca^{2+} from intracellular stores is the binding of ligand to receptor and the transduction of the signal through a guanine nucleotide binding protein that activates a phosphoinositide-specific phospholipase C (PLC). Hydrolysis of phosphatidylinositol-4,5-biphosphate (PIP_2) generates inositol 1,4,5-triphosphate (IP_3), and 1,2-diacylglycerol (DAG) (Berridge 1983); IP_3, in turn, acts as a second messenger and binds to an intracellular receptor on the rough endoplasmic reticulum (RER), releasing Ca^{2+} from intracellular stores (Berridge 1983; Sekar and Hokin 1986). The mechanisms that affect uptake of Ca^{2+} from extracellular sources are less well defined. Two type of channels have been identified in the plasma membrane–hormone-activated channels and voltage-operated channels (Bolton 1979; Rasmussen and Barrett 1984). Since phospholipases release arachidonic acid from the cell membrane, arachidonic acid or its metabolites have been postulated to play a role in opening the hormone-activated channel (Putney et al. 1980; Volpi et al. 1980; Rubin 1982; Sekar and Hokin 1986).

The second product of PIP_2 hydrolysis is 1,2-diacylglycerol, which also functions as a second messenger that has been proposed to activate protein kinase C (Nishizuka 1984). Protein kinase C activation, in turn, is thought to regulate cellular functions through phosphorylation of structural or functional proteins.

Because many inflammatory mediators and growth factors bind to receptors and stimulate phosphoinositide turnover and Ca^{2+} mobilization as part of their signal transduction pathway (Bolton 1979; Rasmussen and Barrett 1984), compounds that inhibit either phospholipase A_2 or C and/or inhibit Ca^{2+} mobilization should have anti-inflammatory activity.

RELATIONSHIP OF MANOALIDE TO PHOSPHOLIPASES AND Ca^{2+}

Manoalide (Fig. 1), a novel marine natural product isolated from the sponge *Luffariella variabilis*, is a potent inhibitor of bee venom (IC_{50} = 0.05 μM) (Jacobs et al. 1985) and cobra venom (IC_{50} = 2 μM) phospholipase A_2 (PLA_2) (Lombardo and Dennis 1985). Glaser and Jacobs (1986, 1987) showed that man-

TABLE 1. SURVEY OF MARINE COMPOUNDS HAVING EFFECTS ON INFLAMMATION AND CALCIUM

Compound	Activity	References
Manoalide	Anti-inflammatory PLC, PLA_2 inhibitor	Jacobs et al. 1985; Glaser and Jacobs 1986; Bennett et al. 1987
Pseudoterosins	Analgesic, anti-inflammatory	Look et al. 1986
Luffariellide	Anti-inflammatory	Albizati et al. 1987
Hyrtial	Anti-inflammatory	Crews et al. 1985
Foliaspongin	Anti-inflammatory	Kikuchi et al. 1983
6-n-tridecyl salicylic acid	Anti-inflammatory	Buckle et al. 1980
Flexibilide	Anti-inflammatory	Buckle et al. 1980
Dendolare	Anti-inflammatory	Buckle et al. 1980
Maitotoxin	Activates VOC-lacrimal glands	Takahashi et al. 1983; Mauduit et al. 1987
Giotoporatoxin	Ca^{2+} channel activator	Qar et al. 1986
Bromo-eudistomin tunicate	Anti-viral activity Ca^{2+} release SR	Nakamura et al. 1986
ω-conatoxin(s)	Blocks N-type Ca^{2+} channels	Rivier et al. 1987; Olivera et al. 1985
Bryostatins	Anti-neoplastic agent Protein Kinase C	Ramsdell et al. 1986; Smith et al. 1985

oalide irreversibly binds to bee venom PLA_2, and suggested that it may represent a novel class of anti-inflammatory agents. See also Mayer and Jacobs (this volume).

Bennett et al. (1987) purified a PI-PLC from guinea pig uterus in which manoalide has an IC_{50} of 2–3 μM. Aswad et al. (1987), using a broken cell preparation of mouse epidermis, showed that manoalide has an IC_{50} of 7 μM. To examine the effect of manoalide on Ca^{2+} mobilization, we chose representative cells and tissues in which the mechanisms of Ca^{2+} mobilization have been established. With quin-2 dye techniques, EGF has been shown to open a hormone-activated plasma Ca^{2+} channel in A431 cells (Moolenaar et al. 1986). In GH_3 cells, Ca^{2+} release from intracellular stores is apparent with TRH stimulation, whereas K^+ depolarization or Bay K8644 opens a voltage-sensitive Ca^{2+} channel that depends on extracellular Ca^{2+} (Schramm et al. 1983; Drummond 1985; Nowycky et al. 1985). Both A431 and GH_3 cells are tumor lines. Therefore, neutrophils and keratinocytes were used to determine the effects on manoalide in non-transformed cells. Change in free cytosolic Ca^{2+} as a function of various agonists was monitored by use of the fluorescent indicator fura-2. In addition, the relationship between Ca^{2+} changes and alterations in PI metabolism was investigated as a function of water-soluble inositol phosphates.

A431 CELLS

Polypeptide mitogens like EGF induce rapid biochemical, metabolic, and early transcriptional changes in responsive cells such as A431. Figure 2A shows changes in $[Ca^{2+}]_i$ after treatment with 100 ng/ml EGF. These cells were selected initially because of the finding by Moolenaar et al. (1986) that EGF raises intracellular Ca^{2+} due to Ca^{2+} entry from extracellular sources. Using quin-2, we also found absolute dependence on the EGF response of medium Ca^{2+}. When using fura-2 as the Ca^{2+}-sensitive dye, a new intracellular Ca^{2+} mobilization component was observed (Fig. 2B, C), as described by Wheeler et al. (1987). Treatment

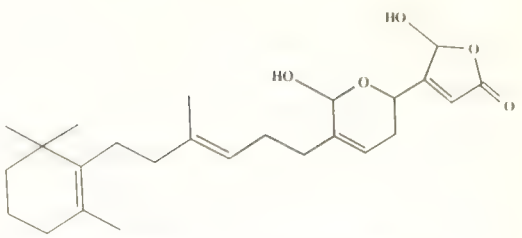

FIGURE 1. Structure of manoalide.

of cells with manoalide completely inhibits both types of Ca^{2+} signals (from outside and release from inside) in a concentration- and time-dependent manner (Fig. 2D, E; IC_{50} = 0.4 μM).

EGF also stimulates PI turnover in A431 cells (Sawyer and Cohen 1981). A detailed study of PI turnover was conducted with A431 cells prelabeled with [^{3}H] myo-inositol. Total inositol phosphates increased linearly for 60 min after addition of EGF (Fig. 3). Separation of the inositol phosphates showed the major product was inositol monophosphate; no increase in IP_3 or IP_2 was detected. Thus, EGF stimulation of A431 cells resulted in uptake of Ca^{2+}, Ca^{2+} release, and increased turnover of phosphoinositides. We could not obtain evidence for a relationship between intracellular Ca^{2+} release and the formation of IP_3. This may be due to limitations in our measurement techniques. Release of Ca^{2+} from intracellular stores could also be due to an alternative signalling system. At concentrations that obliterated either type of $[Ca^{2+}]_i$ response, no effect of manoalide on the production of inositol phosphates was seen (Fig. 3). At higher concentrations (10 × IC_{50}), manoalide inhibited production of inositol phosphates, as expected from studies on inhibition of phospholipases. It would appear that manoalide is able to dissociate inositide turnover and changes in $[Ca^{2+}]_i$ in A431 cells. This would be anticipated if manoalide acts as an inhibitor of Ca^{2+} channels.

GH_3 CELLS

This cell line has been used to study two types of Ca^{2+} responses: the release of Ca^{2+} from intracellular stores, and depolarization-dependent Ca^{2+} entry induced by elevation of medium K^+ (Drummond 1985). This cell model, therefore, allows assessment of the effect of manoalide on a proven IP_3-mediated response and on a voltage-operated plasma membrane channel.

The addition of TRH induced a transient rise in free cytosolic Ca^{2+} ($[Ca^{2+}]_i$) from a basal level of 207 ± 5 nM (n = 43) to 511 ± 87 nM (n = 7) that decayed to almost baseline values in about 5 min (Fig. 4A). One-tenth μM TRH produced a maximal rise in $[Ca^{2+}]_i$ that was independent of medium Ca^{2+} (Fig. 4B). However, in the absence of medium Ca^{2+}, the effect of TRH was short-lived compared to the effect in the presence of medium Ca^{2+}. An increase of medium K^+ induced an increase in $[Ca^{2+}]_i$ (Fig. 4A) that was dependent on the presence of medium Ca^{2+} (Fig. 4B). A concentration of 3.0 μM manoalide for 5 min (or 0.5 μM for 20 min) completely blocked both the hormone and voltage effects on $[Ca^{2+}]_i$ (Fig. 4C). The Ca^{2+} agonist, Bay K8644 (Schramm et al. 1983; Nowycky et al. 1985), when added to GH_3 cells incubated in medium A containing 12 mM K^+, induced a rise in $[Ca^{2+}]_i$ from 208 to 520 nM that was dependent on the presence of medium Ca^{2+} (Fig. 4E). Manoalide inhibited

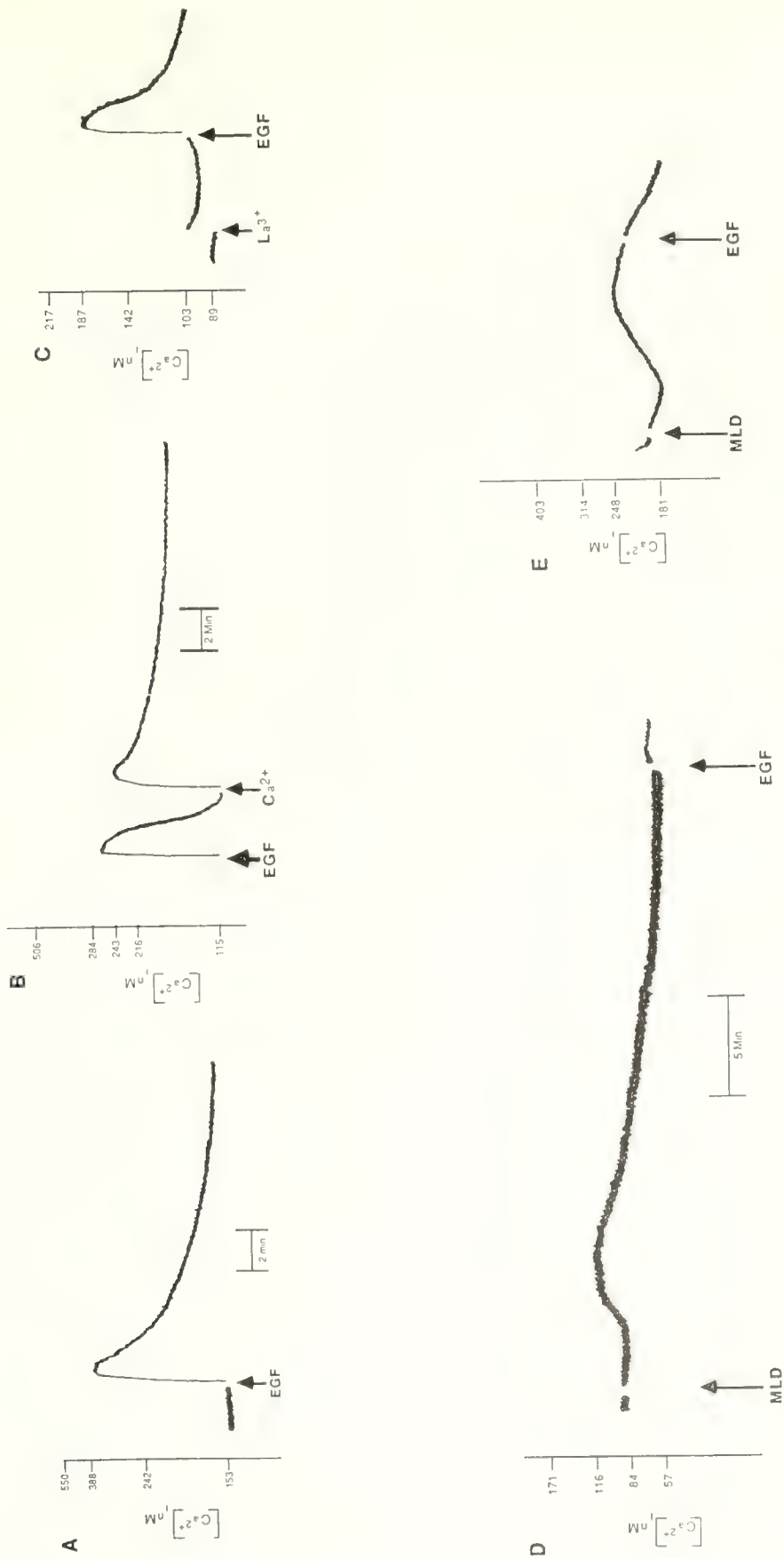

FIGURE 2. Effect of manoalide on the EGF-induced increase in [Ca^{2+}]$_i$. A431 cells were detached from culture plates and incubated with 4 μM fura-2-AM for 15 min at 37°C. (A) A431 cells (7×10^5 cells/ml) were resuspended in medium A (20 mM HEPES buffer pH 7.4 containing 120 nN NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1 mg/ml glucose and 1 mg/ml pyruvate) containing 1.4 mM $CaCl_2$ and incubated at 37°C with continuous stirring in a fluorometer cuvette. Where indicated, cells were stimulated with 100 ng/ml EGF. This experiment is representative of 16 others. (B) A431 cells were added to Ca^{2+}-free medium A and stimulated with 100 ng/ml EGF. Where indicated, 1.4 mM $CaCl_2$ was added to the incubation medium ($n = 3$). (C) A431 cells were resuspended in medium A containing 1.4 mM $CaCl_2$. Where indicated, 100 μM $LaCl_3$ and then 100 ng/ml EGF was added to the incubation medium ($n = 3$). (D) A431 cells were suspended in medium A containing 1.4 mM $CaCl_2$. 0.15 μM manoalide from a stock solution of 1 mM in DMSO was then added. After 30 min of incubation at 37°C, the cells were stimulated with 100 ng/ml EGF ($n = 3$). (E) Experimental procedure as in (D) except that cells were treated with 1.5 μM manoalide for 5 min before stimulation with EGF ($n = 5$). EGF = epidermal growth factor. MLD = manoalide.

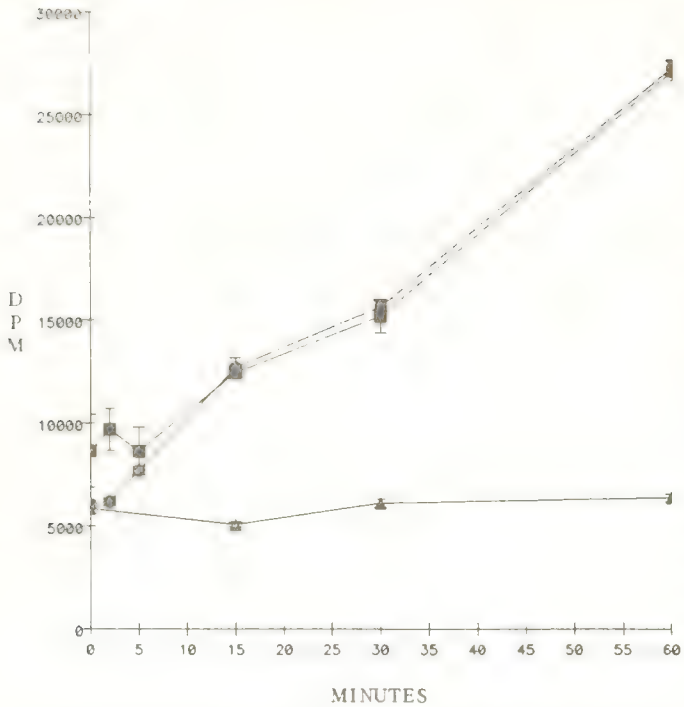

FIGURE 3. Effect of manoalide on EGF stimulated [^{3}H]-IP production in A431 cells. Monolayers of A431 cells were incubated with [^{3}H] inositol (1 μCi/ml) overnight in M199 medium. The cells were then washed twice in medium A containing 5 mM myo-inositol and 1.4 mM $CaCl_2$ and then resuspended in medium A containing 1.4 mM $CaCl_2$ and 5 mM LiCl. Manoalide 1.5 μM (■) or vehicle (●) was then added and incubated at 37°C for 10 min, after which the cells were stimulated with 200 ng/ml EGF. Addition of manoalide (1.5 μM) with no EGF stimulation (▲). At indicated times the reaction was terminated and total water soluble [^{3}H] inositol phosphates determined. Control cells contained 96269 ± 3835 DPM of [^{3}H] label in the lipid fraction. The data shown are mean ± SEM of triplicate determinations.

the effect of Bay K8644 as shown in Figure 4F. The apparent IC_{50}s for TRH, K^+, or Bay K8644 were approximately 1.0 μM.

The action of TRH on the level of IP_3 is shown in Figure 5. The change in IP_3 levels is independent of medium Ca^{2+}, as previously described (Bolton 1979; Volpi et al. 1980; Rasmussen and Barrett 1984). Preincubation with manoalide at 1 μM and 3 μM had no effect on the TRH-induced increment of IP_3, IP_2, and IP. However, at 10 μM there was significant inhibition of IP_3 release. Thus, manoalide inhibited IP_3 formation, presumably due to its effect on phospholipase C at only high concentrations. At concentrations where the $[Ca^{2+}]_i$ increase was blocked, no effect of manoalide on IP_3 levels was found. Therefore, the effect of manoalide on Ca^{2+} signals was dissociated from its effect on phosphoinositide metabolism.

KERATINOCYTES

Leukotriene B_4 (LTB_4) is an inflammatory mediator that is present at elevated levels in psoriasis and cutaneous inflammation (Duell et al. 1986). LTB_4 stimulates DNA synthesis in human keratinocytes (Kragballe et al. 1985). We tested whether LTB_4 could induce changes in $[Ca^{2+}]_i$ in human neonatal foreskin keratinocytes. Compounds that block at this activation step might be useful in inhibiting cutaneous inflammation and hyperproliferation. Foreskins were incubated in trypsin–EDTA overnight, after which epidermis was separated from dermis and single cell suspensions (1×10^6 cells/ml) were made. Addition of LTB_4 induced a rapid, transient rise in $[Ca^{2+}]_i$ (from 122 ± 15 nM, $n = 11$) that decayed to almost baseline levels in 2–3 min (Fig. 6A). The LTB_4-dependent rise in $[Ca^{2+}]_i$ was dose-dependent (10^{-9}–10^{-5} M) with a maximal response at 10^{-6} M. This response was independent of medium Ca^{2+} (Fig. 6B, C). An inactive isomer of LTB_4 did not stimulate Ca^{2+} mobilization (Fig. 6D). Pretreatment of keratinocytes with 1.0 μM manoalide for 5 min completely inhibited changes in $[Ca^{2+}]_i$ (Fig. 7). These results suggest that LTB_4 releases Ca^{2+} from intracellular stores in keratinocytes and that manoalide can inhibit this response.

NEUTROPHILS

In order to determine if the anti-inflammatory activity of manoalide *in vivo* could be due, at least in part, to inhibition of neutrophil activation, we examined Ca^{2+} mobilization and O_2^- production in polymorphonuclear leukocytes (PMNs) in response to various stimuli. The chemotactic agents LTB_4 and fMLP produced rapid, transient increases in $[Ca^{2+}]_i$, as measured by the fluorescent probe fura-2, with maximum responses occurring at concentrations of 10^{-9} M and 10^{-8} M, respectively. These signals peaked within 30 sec and then rapidly fell to basal levels. They were only partially dependent on extracellular Ca^{2+}, consistent with the idea of an initial release from intracellular stores in response to receptor mediated phosphoinositol turnover (for review see Westwick and Poll 1986). Since the generation of phosphatidic acid by fMLP has been reported to be greater than that generated by LTB_4, and the time-course of the IP_3 signal longer, differences in signal-transduction mechanisms have been suggested for these agonists (Omann et al. 1987; Verghese et al. 1987). Manoalide, however, is an equi-potent, non-competitive inhibitor of both agents (IC_{50} approx. 0.15 μM) (Fig. 8). Whether this reflects inhibition of common steps, such as phospholipase C activity or Ca^{2+} channels, within these pathways is currently being investigated. Exposure of PMNs to opsonized zymosan similarly led to an increase in $[Ca^{2+}]_i$, although this response was much slower and completely dependent on the presence of extracellular Ca^{2+}. Manoalide inhibited the zymosan response by only 50%, even at concentrations as high as 1 μM. The reason for manoalide's decreased efficacy against zymosan is not known.

Neutrophils also respond to fMLP by an increase in O_2^- production, as measured by superoxide dismutase inhibitable cytochrome c reduction. Pretreatment of these cells with manoalide led to a dose-dependent inhibition of this response (IC_{50} approx. 0.6 μM). Furthermore, manoalide was observed to be effective (IC_{50} approx. 0.2 μM) in blocking phorbol ester induced O_2^- (Fig. 9). Since TPA's activity is not dependent on extracellular Ca^{2+}, nor does it lead to an increase in free cytosolic Ca^{2+}, manoalide's inhibition of this response is apparently mediated by a mechanism independent of its effect on Ca^{2+} mobilization. It could be due to a direct effect on NADPH oxidase or on some other step beyond protein kinase C activation. These possible mechanisms are currently being examined.

THE EFFECT OF MANOALIDE ON OTHER MEMBRANE FUNCTIONS

In A431 cells, forskolin-stimulated increases in cAMP were not inhibited by 1 μM and 10 μM manoalide. The action of the

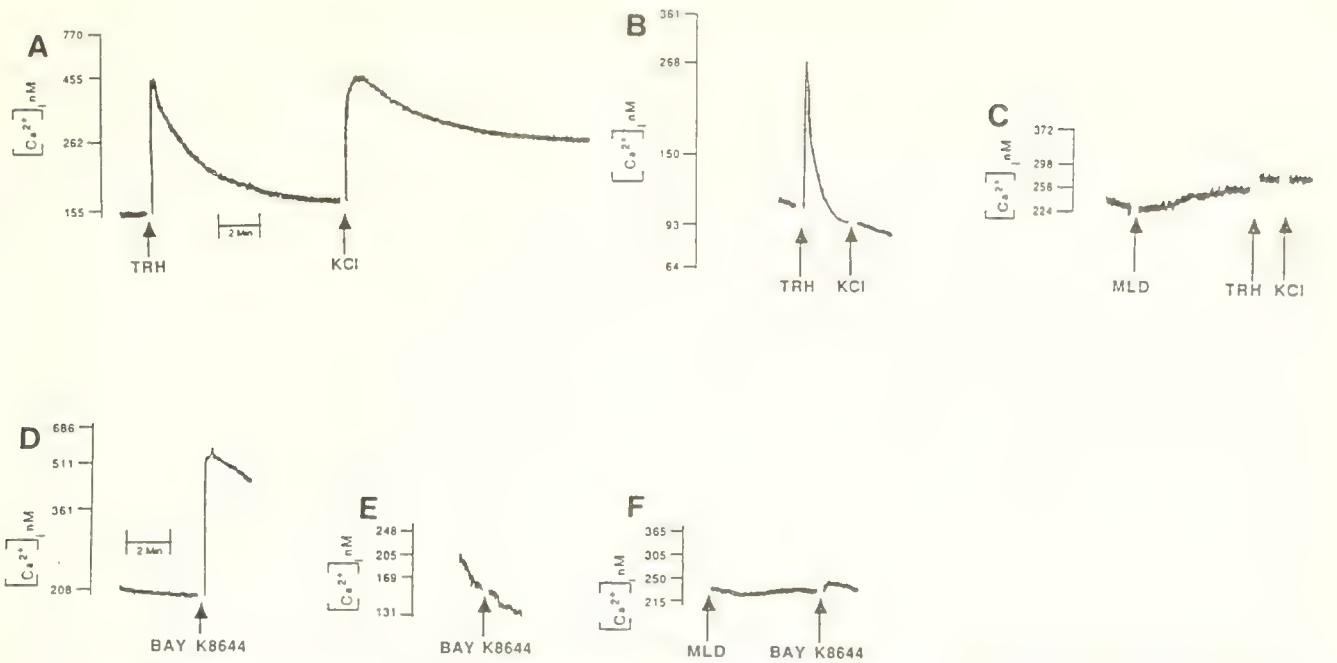

FIGURE 4. Effect of manoalide on TRH-, KCl- and Bay K8644-induced increase in $[Ca^{2+}]_i$ in GH_3 cells. GH_3 cells were detached from culture plates and incubated with 4 μM fura-2-AM for 15 min at 37°C. (A) About 10^5 cells/ml were suspended in medium A and treated as described in Figure 3. Where indicated, 0.1 μM TRH and then 50 mM KCl were added. This experiment is one of six determinations. (B) Fura-2 loaded GH_3 cells were washed once (by centrifugation for 5 min at 300 × *g*) and resuspended in Ca^{2+}-free medium A containing 0.2 mM EGTA. Where indicated, 0.1 μM TRH and 50 mM KCl were added to the incubation medium (n = 3). (C) 3.0 μM manoalide from a 1 mM stock solution in DMSO was added to GH_3 cells suspended in medium A containing 1.4 mM $CaCl_2$. After 5 min of incubation at 37°C, 0.1 μM TRH and 50 mM KCl were added as indicated in the figure (n = 3). (D) GH_3 cells (10^5 cells/ml) were loaded with fura-2 as described above and then suspended in medium A containing 1.4 mM $CaCl_2$ and 12 mM KCl. Where indicated, 1 μM Bay K8644 from a stock solution of 1 mM in ethanol: water (50:50 v/v) was added to the incubation medium. (E) The cells were washed and suspended in Ca^{2+}-free medium A containing 12 mM KCl and 0.2 mM EGTA. Where indicated, cells were stimulated with 1 μM Bay K8644. (F) GH_3 cells suspended in incubation medium similar to that in (D) were incubated with 1 μM manoalide for 5 min before stimulation with 1 μM Bay K8644 (n = 3).

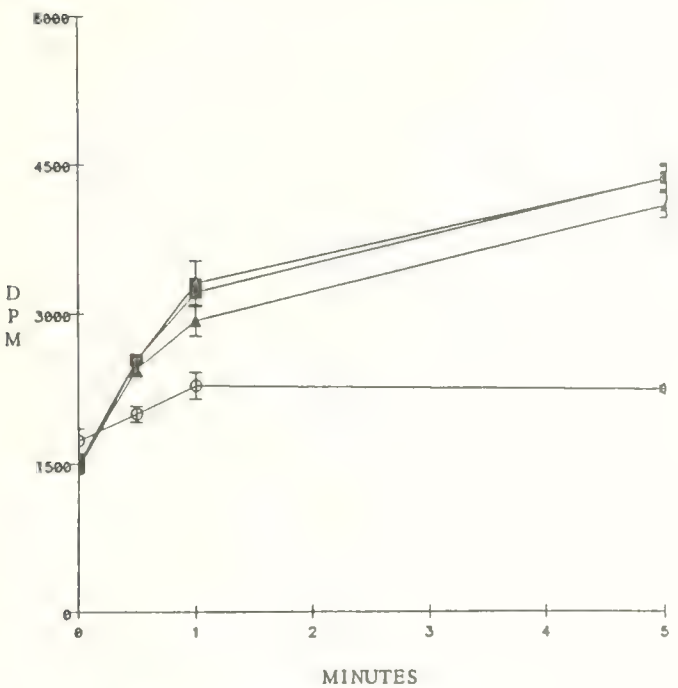

FIGURE 5. Effect of manoalide on TRH mediated IP_3 production in GH_3 cells. Suspensions of GH_3 cells, plated in 150 mm petri dishes at 5 × 10^5 cell/ml in M199, 15% horse serum, 2.5% fetal calf serum were incubated for 72 hr with 4 μCi/ml [^{3}H] inositol. The cells were washed twice in medium A containing 5 mM myo-inositol and 1.4 mM $CaCl_2$, then resuspended in medium A containing 1.4 mM $CaCl_2$ and 5 mM LiCl, and incubated for 10 min at 37°C. Cells were incubated for 5 min at 37°C with either vehicle (●), 1 μM (■), 3 μM (▲) or 10 μM (○) manoalide, before stimulation with 0.1 μM TRH. At indicated times, samples were removed, the reaction was terminated, and IP_3 was separated on Dowex columns as described by Beaven et al. (1984). The results shown are the mean ± SEM of three determinations.

TABLE 2. EFFECT OF MANOALIDE ON FORSKOLIN-STIMULATED CYCLIC AMP PRODUCTION IN A431 CELLS.[a]

Additions	Cyclic AMP nmoles/mg protein[b]		
	Control	Manoalide 1 μM	Manoalide 10 μM
Vehicle	0.08 ± 0.04	0.04 ± 0.003	0.10 ± 0.05
Forskolin 1 μM	0.20 ± 0.07	0.22 ± 0.07	N.D.
10 μM	1.23 ± 0.33	1.34 ± 0.34	N.D.
100 μM	6.94 ± 1.90	7.55 ± 2.00	8.49 ± 0.36

[a] A431 cells were incubated with 0, 1 or 10 μM manoalide for 10 min at 37°C. Then the indicated concentrations of forskolin were added and the incubation at 37°C allowed to proceed for a further 15 min. The reaction was terminated by the addition of 0.5 ml 30% TCA. The supernatant was collected for measurements of cAMP.

[b] Values for control and 1 μM manoalide represent the mean ± SEM of three experiments in which the effect of each drug concentration was determined in triplicate. Values for 10 μM manoalide represent the mean ± SEM of triplicate determinations in one experiment. N.D. = not determined.

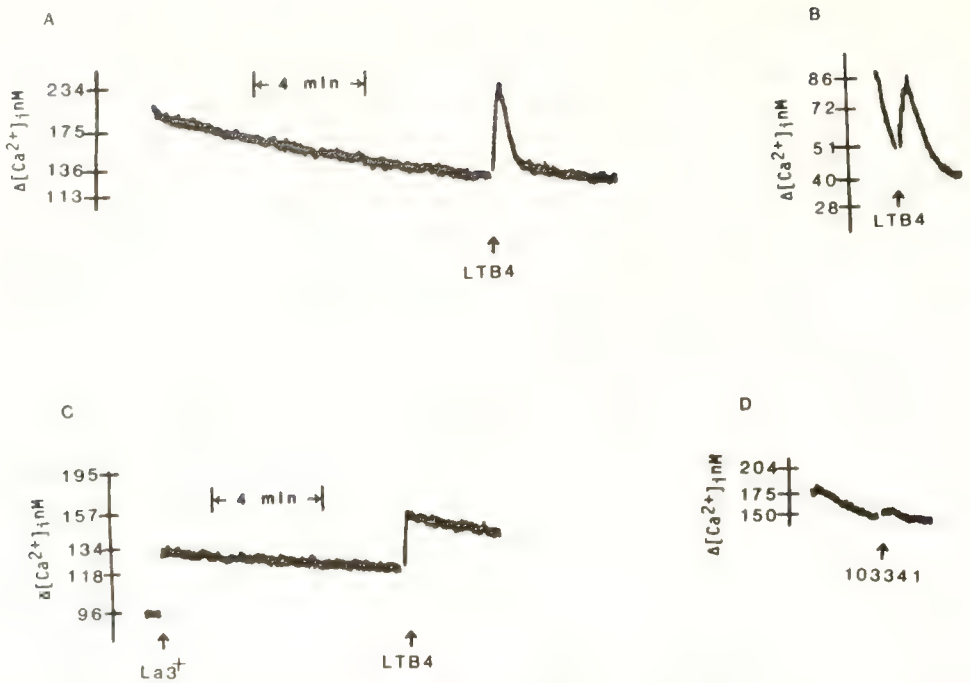

FIGURE 6 Effect of LTB_4 on $[Ca^{2+}]_i$ in human foreskin keratinocytes. Keratinocytes (10^6 cells/ml) loaded with fura-2 (as described in Fig. 3) were stimulated with LTB_4 (10^{-6} M) and $[Ca^{2+}]_i$ was measured. (A) LTB_4 response in medium A containing 1.4 mM Ca^{2+}. (B) LTB_4 response in medium A without Ca^{2+} and containing 0.2 mM EGTA. (C) LTB_4 response in medium A containing 1.4 mM Ca^{2+} and 100 μM La^{3+}. (D) Inactive LTB_4 isomer 103341 10^{-6} M in medium A containing 1.4 mM Ca^{2+}

compound did not extend, therefore, to the membrane bound adenylate cyclase (Table 2).

GH_3 cells respond to depolarization by activation of a voltage-dependent Ca^{2+} channel. Thus, if manoalide altered cell membrane potential, this voltage effect might be blocked. No difference in the uptake and redistribution of the potential sensitive dye $diSC_3$ (5) was noted in control and manoalide-treated GH_3 cells, even in the presence of Ca^{2+}-inhibitory concentrations of manoalide.

CONCLUSIONS

Manoalide is able to block hormone-operated plasma membrane Ca^{2+} pathways, pathways of intracellular Ca^{2+} release, and voltage-operated plasma membrane pathways in a variety of normal and transformed cell types and cellular compartments. The action of manoalide appears to be independent of phosphoinositide metabolism in A431 and GH_3 cells. The activity of manoalide allows some dissection of Ca^{2+} signals from phosphoinositide metabolism, and thus provides a probe for studying Ca^{2+} signalling in inflammation and proliferation. The anti-inflammatory and anti-proliferative activities of manoalide may derive from its Ca^{2+} effects rather than from its effect on phospholipid metabolism. This conclusion must be tempered by the necessity of understanding how manoalide is also inhibiting TPA-induced biological effects, an area of continuing investigation.

ACKNOWLEDGMENTS

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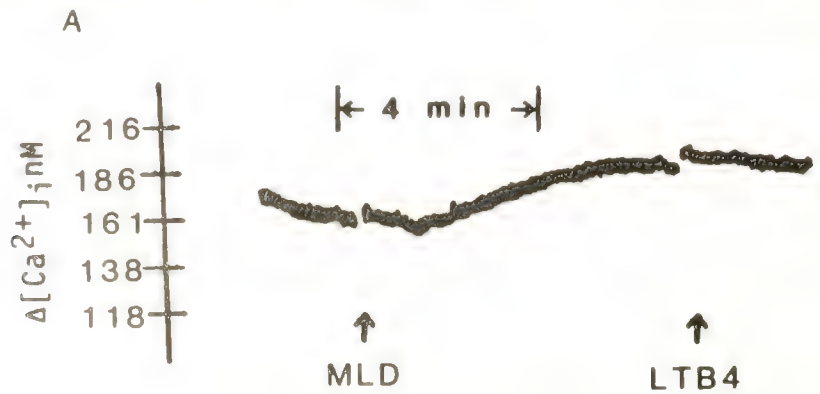

FIGURE 7. Effect of manoalide on LTB_4-stimulated Ca^{2+} mobilization in human foreskin keratinocytes. Cells (1×10^6 cells/ml) were loaded with fura-2 as described in Figure 3 and preincubated with 1 μM manoalide 5 min prior to addition of LTB_4 (1 μM) in medium A containing 1.4 mM $CaCl_2$.

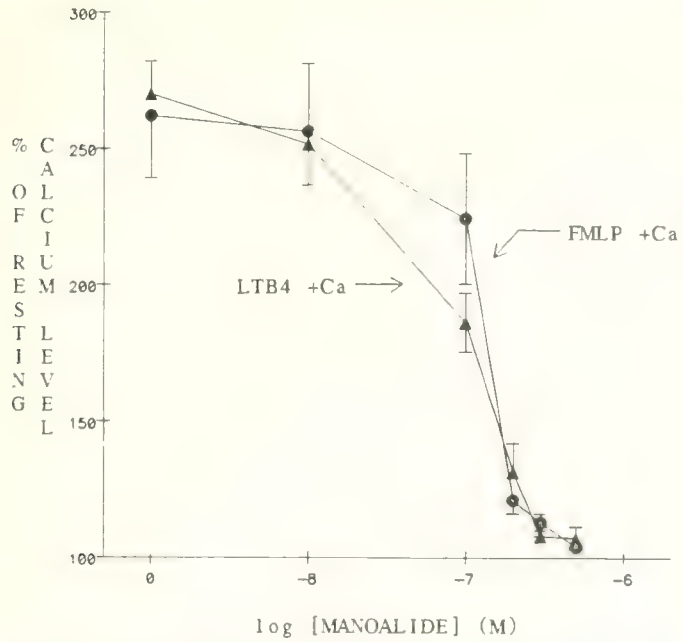

FIGURE 8. Effect of manoalide on fMLP- and LTB_4-stimulated Ca^{2+} mobilization. Human PMNs (5×10^5 cells/ml) were preincubated at 37°C for 5 min with increasing concentrations of manoalide. Intracellular Ca^{2+} levels were determined with the fluorescent probe fura-2 after addition of fMLP (10^{-8} M) or LTB_4 (10^{-9} M). Manoalide produced a dose-dependent (IC_{50} approx. 0.15 μM) inhibition of the Ca^{2+} signal. Values are the mean ± SEM of three experiments.

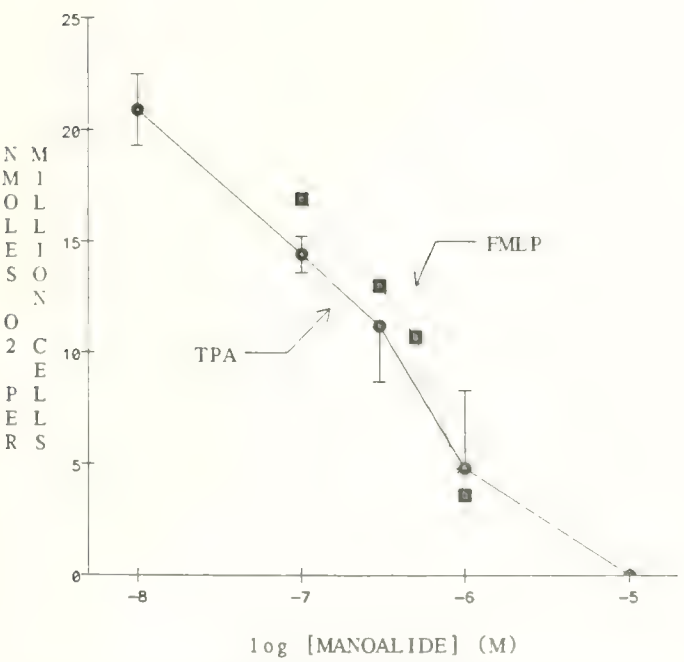

FIGURE 9. Effects of manoalide on fMLP- and TPA-stimulated O_2^- production in human PMNs. Cells (approximately 3×10^6/sample) were pretreated with 5 μg/ml cytochalasin B and/or manoalide for 5 min at 37°C. TPA (5×10^{-7} M) or fMLP (10^{-6} M) were then added to the samples and O_2^- production was determined by measuring SOD inhibitable reduction of cytochrome c. Values are the mean ± SEM of two to five experiments.

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Manoalide: An Antiinflammatory and Analgesic Marine Natural Product

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THE EICOSANOIDS: A BRIEF HISTORICAL BACKGROUND

The eicosanoids, a term applicable to 20-carbon polyunsaturated fatty acids containing a cyclopentane ring, are formed biosynthetically from the three most commonly occurring C_{20} polyunsaturated fatty acids found in invertebrates, vertebrates, and plants: arachidonic (eicosatetraenoic), dihomo-gamma-linolenic (eicosatrienoic) or eicosapentanoic acid (Smith and Borgeat 1985). The discovery of these biologically active fatty acids occurred more than 50 years ago (Kurzok and Lieb 1930) but it was only two decades later that the prostaglandins were structurally elucidated and shown to be a family of compounds with potent biological activity (Bergstrom and Samuelsson 1968). In the middle 1970s, other prostaglandin intermediates, as well as thromboxanes and prostacyclin, were identified (Hamburg et al. 1975; Moncada et al. 1976). In the late 1970s, the lipoxygenase pathway was discovered, thereby establishing two major metabolic pathways for the degradation of arachidonic acid (AA) in living cells (Borgeat and Samuelsson 1979; Corey et al. 1980).

THE EICOSANOIDS IN THE INVERTEBRATES: THEIR PHYSIOLOGICAL ROLES

The discovery of eicosanoids in very primitive phyla suggests that these compounds evolved quite early in metazoans. In 1969, it was reported that an invertebrate, the gorgonian *Plexaura homomalla,* contained 15-epi-prostaglandin A_2 (Weinheimer and Spraggins 1969). Further research has since revealed the presence of prostaglandins in over 100 marine invertebrate species, including the Porifera, Echinodermata, Mollusca, Annelida, Coelenterata, and Arthropoda (Stanley-Samuelson 1987). Eicosanoids seem to participate in fundamental physiological regulatory roles concerning ion flux in bivalves (Dietz 1979; Freas and Grollman 1981; Saintsing and Dietz 1983; Saintsing et al. 1983), behavioral thermoregulation and fever in freshwater and marine arthropods (Casterlin and Reynolds 1978, 1979), hatching in barnacles (Clare et al. 1985), reproduction (Morse et al. 1977; Clare et al. 1986; Kunigelis and Saleuddin 1986), and as second messengers in neuronal cells (Piomelli et al. 1987) of the Mollusca, oocyte maturation in asteroids (Meijer et al. 1984, 1986), and sponge cell aggregation (Rich et al. 1984). The physiological significance of eicosanoids in invertebrates has been further strengthened by the fact that drugs known to be inhibitors of eicosanoid formation have been shown to alter normal biological processes in these animals. Thus, naturally occurring modulators of eicosanoid biosynthesis may exist in these organisms, providing a rationale for the search of naturally occurring eicosanoid inhibitors in marine invertebrates.

THE EICOSANOIDS IN THE PHYSIOLOGY AND PATHOPHYSIOLOGY OF MAMMALIAN SYSTEMS

The eicosanoids are involved in basic physiological processes at the cellular level, and appear to be especially important in various pathophysiological responses in mammals. Prostaglandins, which are metabolically active beginning with the fetus, affect hematopoiesis, are involved in shock-like states, participate in neoplastic diseases, regulate cellular and humoral immunity as well as blood pressure, and play a role in schizophrenia (Cohen 1985). Leukotrienes are increasingly being implicated in mediation of several pathophysiological processes such as generalized or local immune reactions, inflammation, asthma, shock, and trauma (Feuerstein and Hallenbeck 1987). Due to the clinical significance of the eicosanoids, much effort is being directed toward understanding the regulation of AA metabolism, and developing specific inhibitors of prostaglandin and leukotriene biosynthesis. Such inhibitors would have wide therapeutic potential as well as prove invaluable for experimental evaluation of the physiological roles of eicosanoids.

ARACHIDONIC ACID METABOLISM: AN OVERVIEW

The prerequisite for eicosanoid formation in cells is availability of fatty acid. In most cases, this acid is AA, which is found esterified at the sn-2 position of membrane phospholipids and is released by the action of phospholipases. Once released, depending on the tissue, AA is metabolized via two important pathways, the cyclooxygenase pathway and the lipoxygenase pathway. This yields two groups of compounds: the cyclooxygenase products, which consist of the classical prostaglandins as well as prostacyclin and thromboxanes; and the lipoxygenase products, hydroxyperoxy- and hydroxyeicosatetraenoic acids, leukotrienes, and lipoxins. Therefore, the initial and rate-limiting step in the biosynthesis of prostaglandins, leukotrienes, and related compounds is the enzymic liberation of AA from ester pools. The mechanisms involved in regulating release of AA from membrane phospholipids are poorly understood. Multiple enzymatic pathways are most likely involved, such as phospholipase A_2 (Flower and Blackwell 1976; Dennis et al. 1985), the combined action of phospholipase C and a diglyceride lipase (Bell et al. 1979; Lapetina and Cuatrecasas 1979), or other as yet unidentified enzymes or enzyme systems (Ballou et al. 1987). There has been a continuous search for phospholipase inhibitors during the last decade since it is thought that these compounds may help unravel the mechanisms of phospholipid metabolism in normal and diseased cells, as well as have potential clinical use.

MANOALIDE: A PHOSPHOLIPASE A_2 INHIBITOR FROM A MARINE SPONGE

Several years ago, the marine natural product manoalide (MLD), a non-steroidal sesterterpenoid, was isolated from the sponge *Luffariella variabilis* (see De Silva and Scheuer 1980).

The initial pharmacological evaluation of MLD undertaken in our laboratory revealed both an analgesic activity in the phenylquinone-induced writhing assay in mice with an $ED_{50} = 0.36$ mg/kg i.p. (Jacobs et al. 1988), as well as antiinflammatory

TABLE 1. INACTIVATION OF β-BuTX PARALYSIS BY MANOALIDE (PREINCUBATION 1 HR). REPRODUCED FROM DE FREITAS ET AL. 1984 WITH PERMISSION OF BIRKHÄUSER BASEL INC.

Concentration of manoalide ($\times 10^{-8}$ M)	β-BuTX ($\times 10^{-7}$ M)	n	Mean TI_{50} ± SE
0.0	2.4	4	36.0 ± 4.2
0.6	2.4	4	45.2 ± 7.0*
1.2	2.4	4	75.7 ± 16.5*
2.4	2.4	4	137.0 ± 22.2*

* Statistically significant difference relative to β-BuTX alone, $P < 0.05$ unpaired Student's t-test.

activity in the mouse ear inflammation assay (Burley et al. 1982). The observed ED_{50} for MLD, hydrocortisone, and indomethacin in this latter assay were 100 μg, 20 μg, and 250 μg, respectively, indicating that MLD is a potent antiinflammatory agent. Furthermore, indomethacin, a classical cyclooxygenase inhibitor, but not MLD, antagonized the AA-induced inflammation of the mouse ear. This suggested that MLD might not be an inhibitor of the cyclooxygenase pathway similar to indomethacin and other non-steroidal antiinflammatory agents, but that it might

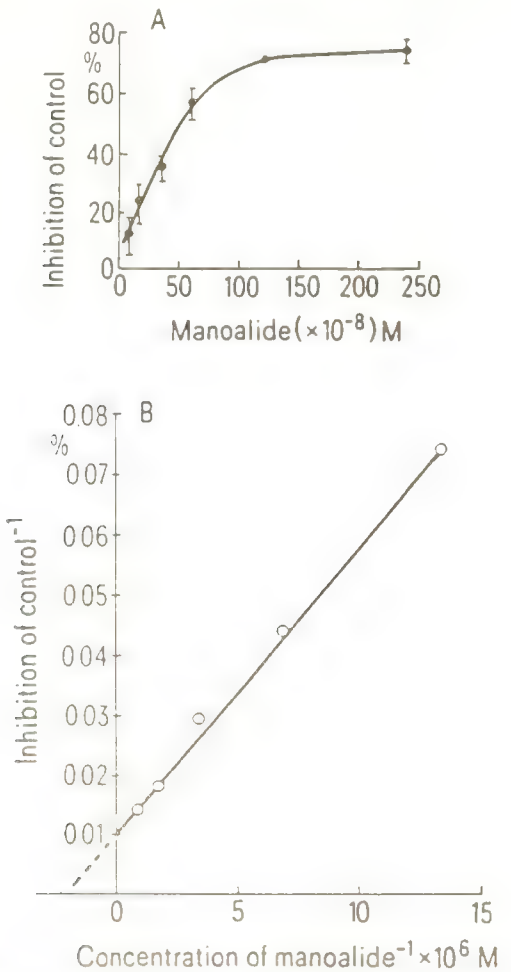

FIGURE 1. Manoalide inactivation of purified phospholipase A_2. Dose–response curve ($n = 6$). A. Percent inhibition of rate of hydrolysis versus manoalide concentration. B. Double reciprocal plot of A. PLA_2 conc. = 0.33 units/ml. Reproduced from De Freitas et al. 1984 with permission of Birkhäuser Basel Inc.

TABLE 2. IRREVERSIBILITY OF THE MLD–PLA_2 COMPLEX. REPRODUCED FROM GLASER AND JACOBS 1986 WITH PERMISSION OF PERGAMON JOURNALS LTD.

Dilution following 60-min preincubation* (μmoles FFA released/min)

	Preincubation time	
MLD (μM)	0 min (0.495 units/ml PLA_2)	60 min (49.5 units/ml PLA_2)
6	0.121	0.035†
50	0.102	0.0023†

Dialysis following 60-min preincubation‡

	% reduction of enzyme activity§	
MLD (μM)	Before dialysis	After dialysis
0.25	86.4	84.9
0.50	91.6	93.0
1.00	92.3	95.0

* Standard assay conditions for the radioassay method were employed. 0-min preincubation—simultaneous addition of PLA_2 and MLD to the substrate mixture. 60-min preincubation—concentrated MLD–PLA_2 mixture preincubated at 41°C and pH 7.4 for 60 min prior to assay. A 100× dilution of the MLD–PLA_2 mixture reduced PLA_2 to the equivalent control concentration (0.495 units/ml) and reduced the MLD concentration to 1/100 of control levels (0.06 and 0.50 μM, respectively).

† Following preincubation, samples were diluted 100× to final assay concentrations of 0.495 units/ml PLA_2, 0.06 and 0.50 μM MLD.

‡ Standard assay conditions for the radioassay method were employed. Before dialysis—MLD–PLA_2 mixtures were preincubated at 41°C and pH 7.4, for 60 min prior to predialysis sampling. After dialysis—the remainder of the MLD–PLA_2 mixture was dialyzed in Spectr/Por MWCO 12,000–14,000 cellulose tubing at 4°C for 24 hr with two buffer changes during the 24-hr period, after which postdialysis samples were assayed. Enzyme activity is reported as percent reduction in enzyme activity as compared to control (without MLD) samples which were treated identically to MLD–PLA_2 mixtures.

§ There was no significant difference between pre- and postdialysis values at $P < 0.05$, Student's t-test, $n = 3$.

act prior to the cyclooxygenase step in prostaglandin synthesis, possibly at a site prior to AA release.

The next series of experiments undertaken in our laboratory to test this hypothesis demonstrated that MLD prevented the neurotoxic action of beta-bungarotoxin, a potent snake venom, on a rat phrenic nerve-diaphragm preparation. As is shown in Table 1, increasing concentrations of MLD caused a statistically significant increase in the TI_{50} (average time to reach 50% paralysis) (De Freitas et al. 1984). The observed inactivation of beta-bungarotoxin prompted us to determine if MLD would also inactivate a purified source of phospholipase A_2 (PLA_2), since the presence of this enzyme as a subunit in beta-bungarotoxin has been implicated in its toxicity (Kondo et al. 1978). When MLD was pre-incubated for 1 hr with purified PLA_2, the subsequent hydrolysis of phosphatidylcholine was impeded as shown in Figure 1. When the percent inhibition of reaction velocity was plotted against MLD concentrations (Fig. 1A), a typical saturation effect was observed. A double reciprocal plot of these data (Fig. 1B) proved to be linear, implying the presence of a homogenous population of receptors. The apparent K_D observed for MLD (4.8×10^{-7} M) indicated that this compound was in fact a potent inactivator of PLA_2 (De Freitas et al. 1984).

MLD seems therefore to be a new type of PLA_2 inhibitor. Inactivators of PLA_2 are relatively few, the only other compounds generally known to inactivate PLA_2 are mepacrine, p-bromophenacyl bromide (BPB), and some of its analogs. BPB has been shown to inactivate PLA_2 from pancreatic tissues (Bon-

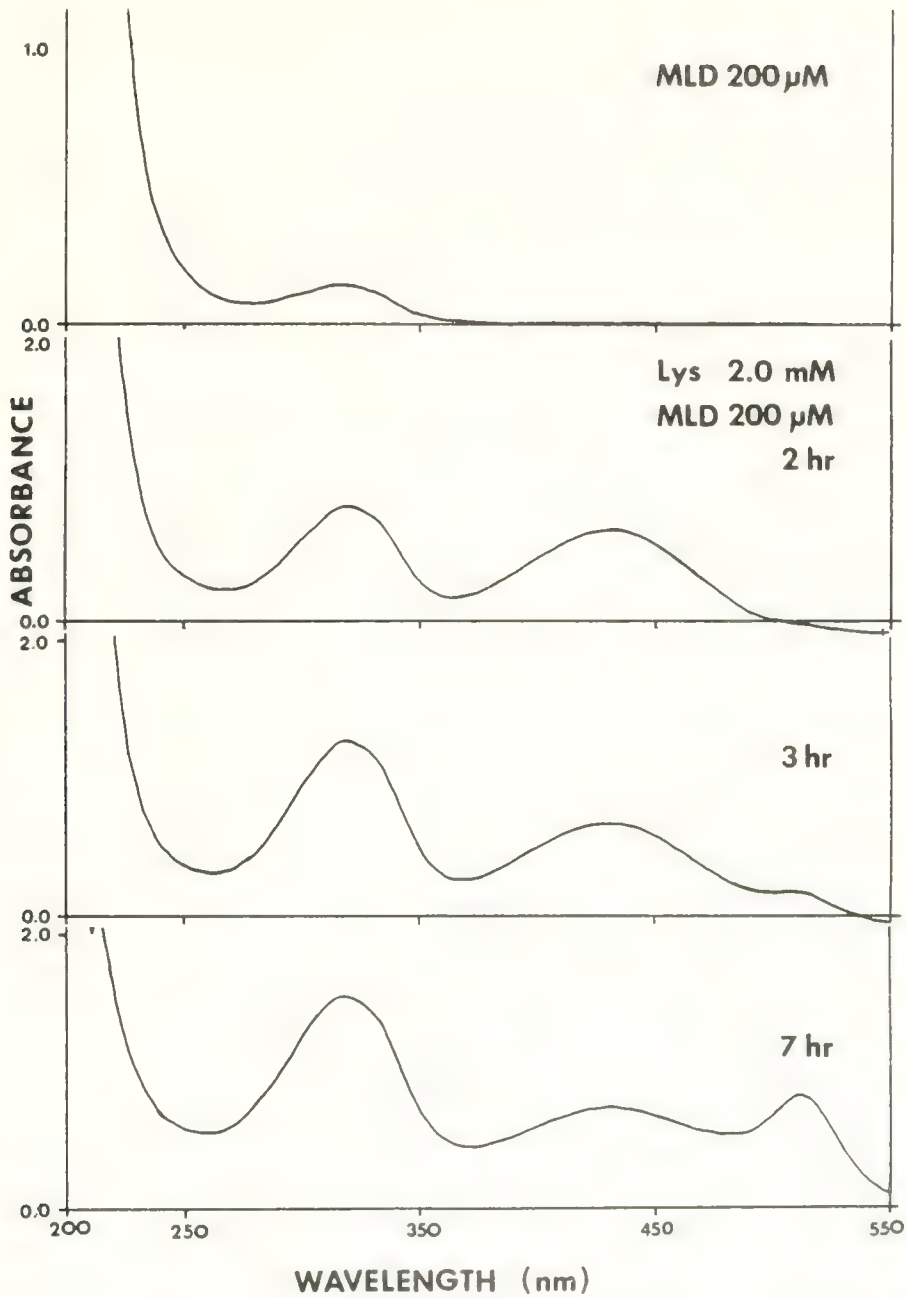

FIGURE 2. Scanning spectrophotometry of the MLD–Lys reaction. MLD (200 μM) was incubated with Lys (2.0 mM) in MeOH (spectral grade reagent) at 41°C. Spectral scans were performed at t = 0, 2, 3, and 7 hr at a scanning speed of 200 nm/min. Reference cells contained Lys (2.0 mM) in MeOH. Reproduced from Glaser and Jacobs 1987 with permission of Pergamon Journals Ltd.

sen et al. 1972) and numerous snake venoms (Yang and King 1980), while mepacrine is an anti-malarial agent that decreases release of AA in experimental models (Vargaftig et al. 1980; Vadas 1982). Both compounds require high concentrations (mM) to inactivate or reduce PLA_2 activity and, as such, are limited in their use as pharmacological probes.

THE MOLECULAR PHARMACOLOGY OF MANOALIDE

We undertook further studies to determine the mechanism of inactivation of bee venom PLA_2 by MLD. These showed MLD to be irreversible and extremely potent with an IC_{50} = 0.05 μM for approximately 25 nM bee venom PLA_2 (Glaser and Jacobs 1986). Additional studies showed the formation of a drug–enzyme complex that is pH-dependent, reaching a maximum at pH 8.0. It is also time-dependent, concentration-dependent, and Ca^{2+}-independent (Glaser and Jacobs 1986). As is shown in Table 2, dissociation of MLD from PLA_2 was not evident following dilution or dialysis, suggesting that inactivation may be irreversible (Glaser and Jacobs 1986). MLD produced a chromophore (λ_{max} = 437 nm) when incubated with bee venom PLA_2 (Fig. 2). A similar chromophore could also be produced by the reaction of MLD with monomeric lysine, cysteine, or tryptophan, but not with their N-alpha-amino-blocked analogs (Glaser and Jacobs 1986).

TABLE 3. MANOALIDE REACTIVITY WITH FREE AMINO ACIDS.* REPRODUCED FROM GLASER AND JACOBS 1987 WITH PERMISSION OF PERGAMON JOURNALS LTD.

Amino acid	Concentration (mM)	Reactivity ratio
Lys	2.0	1.00
Gly	4.0	0.17
Cys	2.0	3.00
Trp	2.0	0.58
Orn	2.0	1.41
N-α-Acetyl-Lys	2.0	0.08
N-α-Acetyl-Lys	4.0	0.00
N-ε-Acetyl-Lys	2.0	0.20
N-ε-Acetyl-Lys	4.0	0.00
N-α-Acetyl-Cys	2.0	0.20
N-α-Acetyl-Cys	4.0	0.01
N-*t*-BOC-*S*-benzyl-Cys	2.0	0.20
N-*t*-BOC-*S*-benzyl-Cys	4.0	0.13
N-*t*-BOC-Trp	2.0	0.26
Glutathione		
Reduced	2.0	6.97
Oxidized	2.0	0.49

* MLD (200 μM) was preincubated for 120 min at 41°C, in 10 mM HEPES, 1 mM $CaCl_2$ at pH 7.4, with 2.0 mM or 4.0 mM free amino acid. Reactivity ratio is the absorbance of the amino acid/absorbance of Lys at 437 nm.

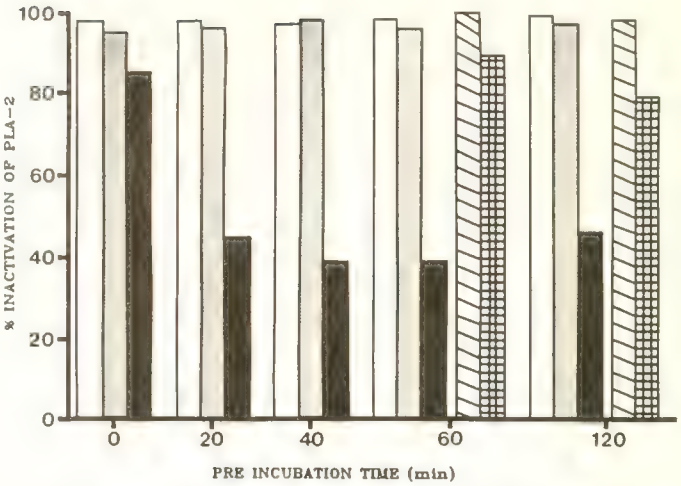

FIGURE 3. Effects of Lys and Cys on the ability of MLD to inactivate bee venom PLA_2. MLD (100 μM) was preincubated with buffer (□), 1.0 mM of Lys (□), Cys (▩), N-alpha-acetyl-Lys (□) or N-alpha-acetyl-Cys (▩) at 41°C (pH 7.4); aliquots were removed at the indicated times, added to an equal volume of PLA_2 (5.0 μM), incubated for 60 min at 41°C (pH 7.4), and assayed for PLA_2 activity. Final concentrations assayed were: MLD, 0.5 μM; Lys, Cys and N-alpha-amino-modified analogs, 5.0 μM; and PLA_2, 25 nM. The standard error of the mean was 10% or less of the mean for each data point ($n = 4$). Reproduced from Glaser and Jacobs 1987 with permission of Pergamon Journals Ltd.

In order to determine the binding site of MLD on PLA_2, the possible correlation between chromophore production and the specific amino acid residue modified on PLA_2 by MLD was investigated. The reaction of MLD with free or N-alpha-amino-modified amino acids was observed. Although lysine, cysteine, and tryptophan produced a significant chromophore (Table 3), they did not affect PLA_2 activity (Fig. 3).

Furthermore, polymers of lysine prevented MLD from inhibiting PLA_2, but monomeric lysine did not. The most active polymer appeared to be a tetralysine, with a degree of selectivity when the lysine residues were in the 1,4 arrangement (Fig. 4). It was concluded from these studies that MLD reacts with bee venom PLA_2 and polymers of lysine by an ordered reaction, rather than by a random reaction as would be expected if the drug reacted with all available lysine residues (Glaser and Jacobs 1986, 1987).

Based on these studies, we pursued the possibility that there is a specific binding site(s) for MLD on bee venom PLA_2. In recent experiments we found that the only change in amino acid content of treated bee venom is an apparent loss of three of the 11 lysine residues in the venom. When we modified the lysine residues with [^{14}C] maleic anhydride, MLD treated venom protected labeling of three lysines. When bee venom PLA_2 was cleaved with cyanogen bromide, MLD was shown to be bound to three fragments isolated by reverse phase HPLC. The most intense peak corresponded to amino acid residues 81-128, as determined by gas-phase microsequence analysis. Amino acid sequence analysis of this fragment showed the presence of a lysine-X-X-lysine (1,4) peptide arrangement that is in close proximity to the active site core (histidine^{34}-aspartase^{35}). We have postulated that the MLD binding site may correspond to positions of the amino acid sequence necessary for substrate binding (Glaser and Jacobs 1988).

Some 40 analogs of MLD have been isolated thus far. We have undertaken a structure activity study with John Faulkner's group to determine the reactive sites on the MLD molecule. Thus far, reversible analogs have been identified and their structure elucidated (Albizati et al. 1987; Kernan et al. 1987).

THE BIOLOGICAL SIGNIFICANCE OF THE PHOSPHOLIPASES

PLA_2 enzymes are ubiquitous in nature, being natural cellular constituents, a component of many venoms, a secreted enzyme necessary for digestion in many animals, and important in the physiology of marine organisms (Meijer et al. 1984). The ubiquitousness of this enzyme appears to relate to its function and the relative homology of the hydrolytic site. Thus it represents a pleomorphic class of enzymes in certain respects—i.e., specific amino acid sequences involved in the catalytic mechanism are redundant in various sources of the enzymes as well as the calcium binding sites. Non-homologous PLA_2s differ in the side chains surrounding the active site, which allow hydrophobic interactions with lipids (Dijkstra et al. 1981). These side chains, in our view, offer many opportunities for highly specific drug interactions. Drugs bind to these sites presumably by ordered reactions involving at least two amino acids, and require a specific intramolecular distance. If a particular high-affinity substrate binding site were found to be unique to only a few sources of PLA_2, then the inhibitor would become a powerful tool to define PLA_2 function in that particular PLA_2 source.

MANOALIDE AS A DRUG TO DEFINE SUBSTRATE BINDING SITES ON PHOSPHOLIPASE A_2

MLD partially fulfills the criteria proposed (De Freitas et al. 1984; Glaser and Jacobs 1986, 1987, 1988; Albizati et al. 1987; Kernan et al. 1987) in that it is more active against bee venom PLA_2 than cobra venom PLA_2. Furthermore, it blocks hydrolysis of phosphatidylethanolamine and phosphatidylcholine by bee venom to an equal degree, but blocks phosphatidylcholine hydrolysis only by cobra venom PLA_2 (Lombardo and Dennis 1985). Therefore, there may be at least two types of high-affinity substrate binding sites on cobra venom but only one type on bee venom. We are currently investigating new analogs that

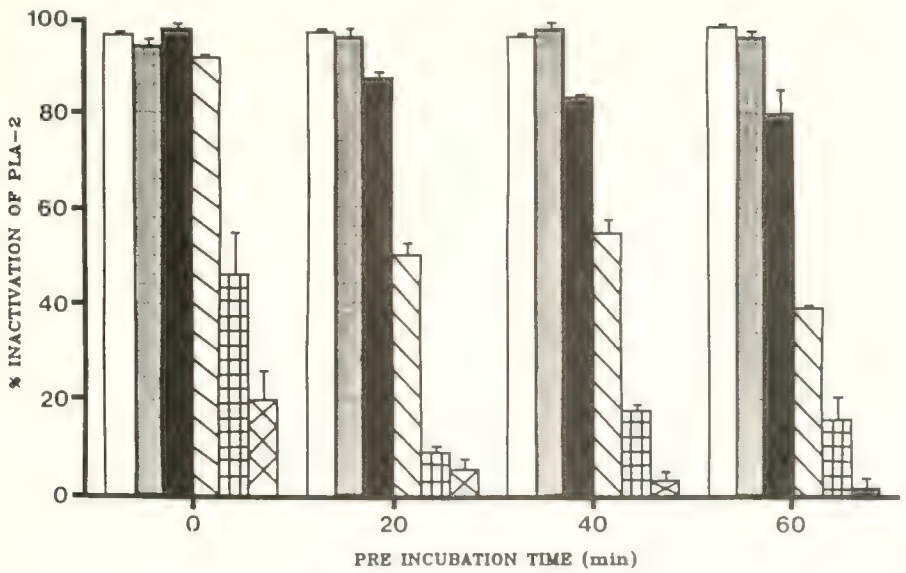

FIGURE 4. Effects of Lys peptides on the ability of MLD to inactivate PLA_2. MLD (100 μM) was preincubated with buffer (□), 1.0 mM Lys (□), or equimolar L_2 (■), L_3 (□), L_4 (▦), and poly-L-Lys (⊠) at 41°C (pH 7.4); aliquots were removed at 20 min intervals, added to an equal volume of PLA_2 (5 μM), incubated for 60 min at 41°C (pH 7.4), and assayed for PLA_2 activity. Final concentrations assayed were: MLD, 0.5 μM; Lys, 5.0 μM; Lys peptides, 0.5 μM; and PLA_2, 25 nM (n = 3). Reproduced from Glaser and Jacobs 1987 with permission of Pergamon Journals Ltd.

initially appear more specific. In contrast, based on current information, active hydrolytic site targeted drugs possibly lack the pharmacological specificity that may potentially be found in drugs that react with high-affinity substrate binding sites. Thus we postulate that this would be a rational approach to develop novel selective inhibitors of the ubiquitous PLA_2 enzymes.

MANOALIDE AS A DRUG TO INVESTIGATE THE *IN VIVO* ROLE OF PHOSPHOLIPASES

Thus far there are few, conflicting, reports on the effects of MLD on intracellular phospholipases. MLD has been shown to be a poor inactivator of crude cytosolic fractions containing PLA_2 activity from guinea pig lung and uterus, rat basophilic leukemia cells, and a smooth muscle-like cell line (Bennett et al. 1987). However, a phosphatidylinositol (PI) specific PLC purified from guinea pig uterus was shown to be more sensitive than cytosolic PLA_2 to inactivation by MLD (IC_{50} = 1.5 μM) (Bennett et al. 1986). In contrast, when MLD was tested on rabbit polymorphonuclear leukocytes, a cell relevant to the inflammatory process, it strongly inactivated PLA_2 activity (IC_{50} = 3 μM), whereas PLC activity from the same cell type was not inhibited (Meade et al. 1986).

MLD has also been shown to block intracellular and extracellular calcium mobilization in several cell types without affecting phosphoinositide metabolism, providing additional evidence for absence of PLC inhibition in some cell types (Wheeler et al. 1987). Thus the currently available evidence seems to suggest that MLD inhibits a phospholipase, possibly a PLA_2, and that it blocks calcium channels in cell types relevant to the inflammatory process.

THE MACROPHAGE AS A MODEL FOR THE STUDY OF PHOSPHOLIPID METABOLISM

The deacylating reactions by which phospholipases release esterified AA from phospholipids for oxygenation by the cyclooxygenase and 5-lipoxygenase pathways in mouse peritoneal macrophages are being extensively investigated. However, the complete details of this process are not yet clear. Phospholipase A_1 (PLA_1), PLA_2, PLC, and lysophospholipases have been proposed as the lipolytic enzymes for AA release in mouse peritoneal macrophages (Dennis et al. 1985; Moscat et al. 1986). Thus far, two distinct PLA_2 enzymes have been identified and characterized in macrophages: a PLA_2 active at pH 4.5, calcium-independent, and probably of lysosomal origin, capable of hydrolyzing phosphatidylethanolamine and phosphatidylcholine; a second PLA_2 active at pH 8.5, calcium-dependent and possibly membrane-bound (Wightman et al. 1981*a*, *c*). In addition, three other phospholipases have been described: a PLC active at neutral pH, calcium-dependent, and highly specific for PI (Wightman et al. 1981*b*); a PLA_1 active at pH 4.2 and Ca^{2+}-independent (Dennis et al. 1985); and a phospholipase C-diglyceride lipase system (Moscat et al. 1986).

EFFECT OF MANOALIDE ON AA RELEASE IN THE MACROPHAGE

In order to study the effect of MLD on the release of AA from macrophage phospholipids, the effects of MLD on the release of [^{3}H]AA from mouse peritoneal macrophages stimulated with PMA was investigated. MLD inhibited the release of [^{3}H]AA, a maximum of 37% inhibition being observed with 0.05 μM MLD. This observation suggested that MLD decreases the availability of AA, which would be expected to produce a corresponding reduction in release of prostaglandins and leukotrienes (Bonney et al. 1980; Rouzer et al. 1980; Humes et al. 1982).

EFFECT OF MANOALIDE ON AA METABOLISM IN THE MACROPHAGE

MLD produced a dose-dependent inhibition of PGE_2 release when mouse peritoneal macrophages were stimulated with PMA (Fig. 5a), A23187 (Fig. 5b), and zymosan (Fig. 5c). MLD also

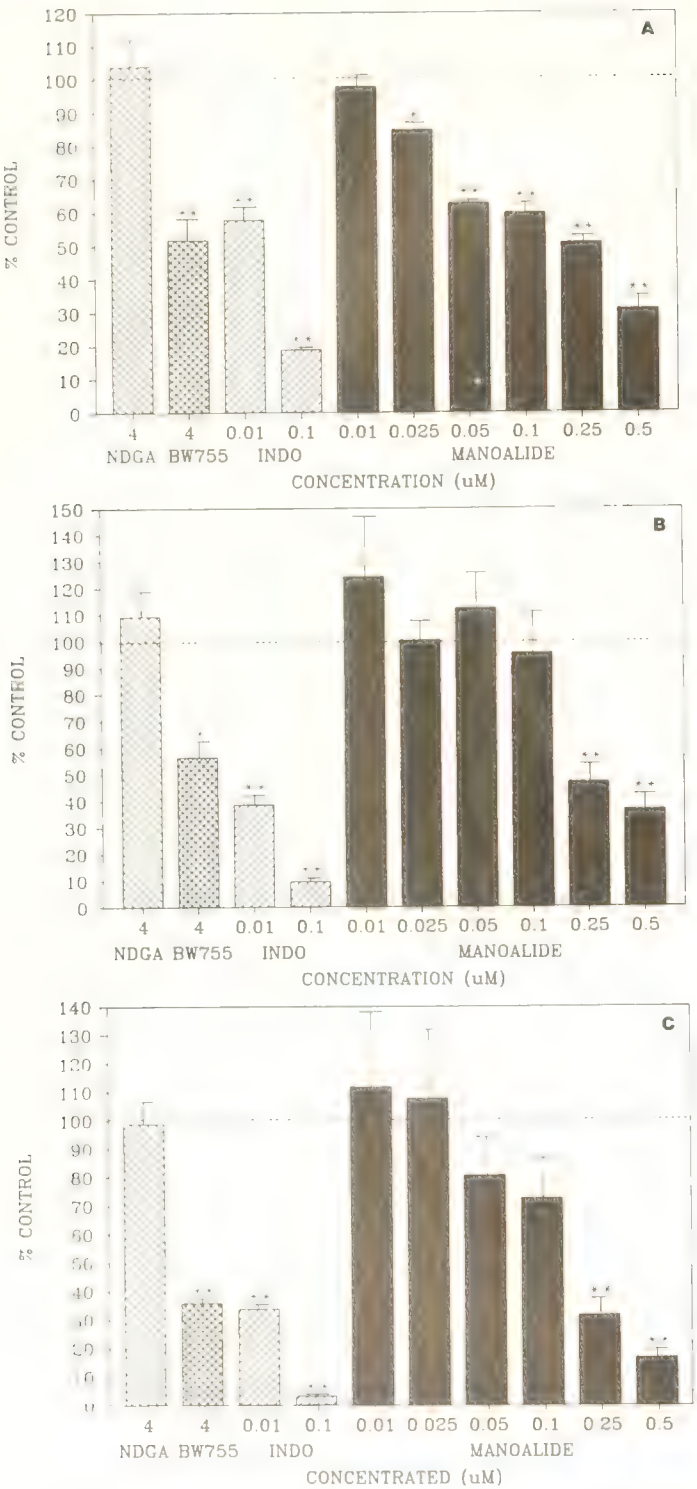

FIGURE 5. a. Effect of MLD on PGE_2 production by mouse peritoneal macrophages stimulated with PMA (1 μM). BW755C (4 μM) and indomethacin (0.01, 0.1 μM) inhibited PGE_2 ($P < 0.01$). MLD (0.025 μM) inhibited PGE_2 ($P < 0.05$); MLD (0.05, 0.1, 0.25, 0.5 μM) inhibited PGE_2 ($P < 0.01$). The approx. IC_{50} for MLD was 0.25 μM. Control PGE_2 production = 338 ng/mg protein. b. Effect of MLD on PGE_2 production by mouse peritoneal macrophages stimulated with A23187 (1 μM). BW755C (4 μM) inhibited PGE_2 ($P < 0.05$); indomethacin (0.01, 0.1 μM) and MLD (0.25, 0.5 μM) inhibited PGE_2 ($P < 0.01$). The approx. IC_{50} for MLD was 0.23 μM. Control PGE_2 production = 118 ng/mg protein. c. Effect of MLD on PGE_2 production by mouse peritoneal macrophages stimulated with zymosan (50 μg/ml). BW755C (4 μM), indomethacin (0.01, 0.1 μM), and MLD (0.25, 0.5 μM) inhibited PGE_2 ($P < 0.01$). The approx. IC_{50} for MLD was 0.18 μM. Control PGE_2 production = 201 ng/mg protein. Reproduced from Mayer et al. 1988 with permission of Waverly Press Inc.

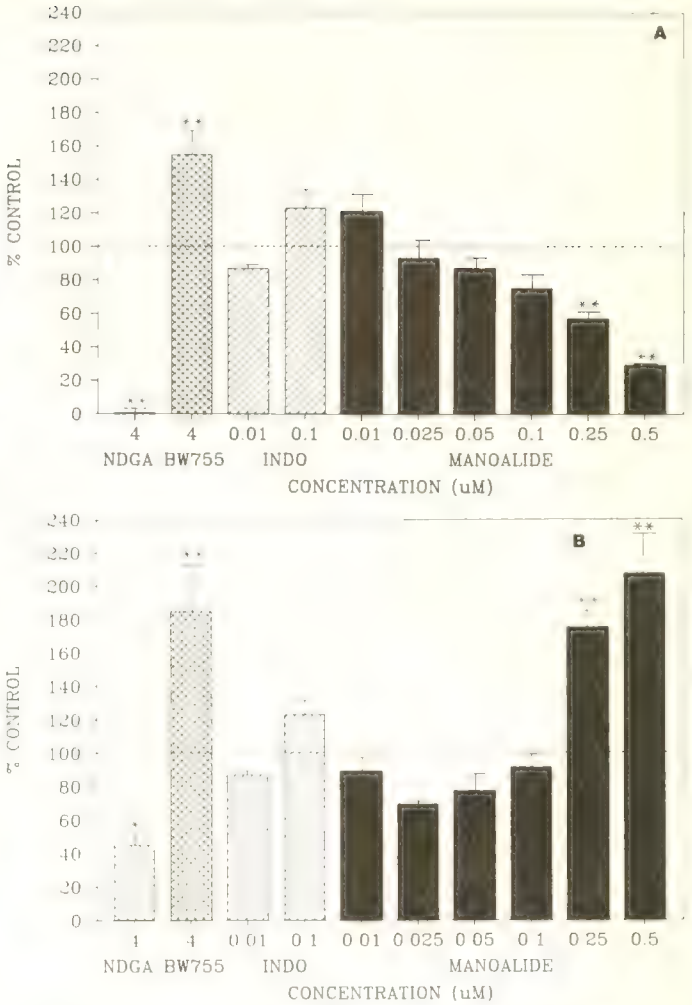

FIGURE 6. a. Effect of MLD on LTC_4 production by mouse peritoneal macrophages stimulated with A23187 (1 μM). BW755C (4 μM) enhanced LTC_4 ($P < 0.01$). NDGA (4 μM) and MLD (0.25, 0.5 μM) inhibited LTC_4 ($P < 0.01$). The approx. IC_{50} for MLD was 0.25 μM. Control LTC_4 production = 704 ng/mg protein. b. Effect of MLD on LTC_4 production by mouse peritoneal macrophages stimulated with zymosan (50 μg/ml). NDGA (4 μM) inhibited LTC_4 ($P < 0.05$). However BW755C (4 μM) and MLD (0.25, 0.5 μM) enhanced LTC_4 ($P < 0.01$). Control LTC_4 production = 254 ng/mg protein. Reproduced from Mayer et al. 1988 with permission of Waverly Press Inc.

produced a dose-dependent inhibition of LTC_4 release when murine peritoneal macrophages were stimulated with A23187 (Fig. 6a). However, LTC_4 production was enhanced by MLD when the cells were stimulated with zymosan (Fig. 6b). Simultaneous inhibition of PGE_2 and enhancement of LTC_4 release upon zymosan stimulation suggested that MLD was possibly inhibiting the cyclooxygenase pathway.

It has been reported that in the absence of phagocytic or pharmacologic stimuli, resting mouse peritoneal macrophages will metabolize exogenously supplied AA into PGI_2, PGE_2, and hydroxyeicosatetraenoic acids (Scott et al. 1982). Although MLD

enhanced the conversion of exogenously added AA to PGE_2 at low concentrations (0.01–0.05 μM), thereafter a dose-dependent inhibition of PGE_2 release was observed, thus providing preliminary evidence that MLD may be also affecting the cyclooxygenase pathway at higher concentrations (Fig. 7)

PHOSPHOLIPID METABOLISM IN ZYMOSAN-TREATED MACROPHAGES: THE USE OF MANOALIDE IN ELUCIDATING THE PATHWAYS LEADING TO AA RELEASE

In zymosan-stimulated macrophages, the release of AA appears to be either the deacylation of phosphatidylcholine (Bonney et al. 1978; Hsueh et al. 1979; Dennis et al. 1985) with a concomitant decrease in the cellular activity of pH 4.5 PLA_2 (Wightman et al. 1981*a*), or the degradation of phosphatidylinositol (PI) (Emilsson and Sundler 1984, 1986). Although pH 4.5 PLA_2 and pH 8.5 PLA_2 have no activity against PI (Wightman et al. 1981*a*), a PI-specific PLC has been reported, active at neutral pH and calcium dependent (Wightman et al. 1981*b*). Zymosan stimulation induces extensive degradation of PI, and gives rise to the hydrolysis products inositol 1-phosphate and inositol 1,4-biphosphate, and the deacylation products lysophosphatidylinositol, glycerophospho-inositol, and monoacylglycerol via a postulated PLA_1 and lyso-phospholipase PI-specific pathway (Emilsson and Sundler 1984).

If MLD inhibits PLA_2 in mouse peritoneal macrophages, as has been shown for rabbit polymorphonuclear phagocytes (Meade et al. 1986), and blocks Ca^{2+} channels, as has been reported in spleen cells (Wheeler et al. 1987), in zymosan-stimulated macrophages, AA release and metabolism into eicosanoids might still be possible by PI-specific PLC, PLA_1, and lysophospholipase pathways as suggested by Emilsson and Sundler (1984). There is evidence of an alternative mechanism for AA release via a PLC-diglyceride lipase mechanism in mouse peritoneal macrophages (Moscat et al. 1986). Definitive elucidation of the alternative mechanisms for AA release from zymosan-stimulated macrophage phospholipids might benefit from further investigation of phospholipid metabolism in the presence of MLD.

PHOSPHOLIPID METABOLISM IN A23187-TREATED MACROPHAGES: THE USE OF MANOALIDE IN ELUCIDATING THE PATHWAYS LEADING TO AA RELEASE

A23187 induces a rapid breakdown of PI in the presence of Ca^{2+} via a PI-specific PLC mechanism. The main water-soluble products are inositol 1,4-biphosphate and diacylglycerol. The diacylglycerol formed by this pathway then stimulates protein kinase C, leading to the activation of a PLA_2 pathway to yield AA (Emilsson and Sundler 1984). A23187 has not been shown to stimulate the deacylation of phosphatidylinositol-4-phosphate or phosphatidylinositol 4,5-biphosphate, so does not seem to release AA via an alternative PLA_1 and lysophospholipase PI-specific mechanism, as has been proposed for zymosan stimulation of macrophages (Emilsson and Sundler 1984). If MLD inhibits both PLA_2 and Ca^{2+} channels in murine peritoneal macrophages, in A23187-stimulated macrophages the release of AA would be inhibited and consequently an inhibition of cyclooxygenase and lipoxygenase products would occur. We have observed both results in our experiments.

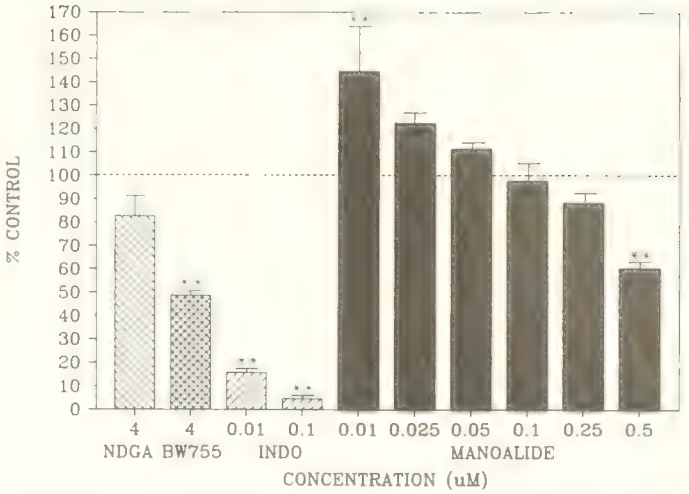

FIGURE 7. Effect of MLD on PGE_2 production by mouse peritoneal macrophages stimulated with AA (2 μM). BW755C (4 μM), indomethacin (0.01, 0.1 μM), and MLD (0.5 μM) inhibited PGE_2 ($P < 0.01$). However, MLD (0.01 μM) enhanced PGE_2 ($P < 0.01$). The IC_{50} for MLD was >0.5 μM. Control PGE_2 production = 343 ng/mg protein.

PHOSPHOLIPID METABOLISM IN PMA-TREATED MACROPHAGES: THE USE OF MANOALIDE IN ELUCIDATING THE PATHWAYS LEADING TO AA RELEASE

PMA is a potent activator of AA release. This response, however, seems to vary with the cell type. It has been observed in the MDCK cell line (Daniel et al. 1981) and macrophages (Brune et al. 1978; Bonney and Humes 1984), but could not be observed in guinea pig neutrophils (Takenawa et al. 1985) or human platelets (Lapetina 1985). PMA stimulation of macrophages results in a greater than 80% release of AA from PI, accompanied by the accumulation of the deacylation products lysophosphatidylinositol and glycerophosphoinositol (Emilsson and Sundler 1986). PMA has structural similarities to diacylglycerol, and will bind and activate protein kinase C, a process that is Ca^{2+}-dependent (Nishizuka 1984). Thus, if MLD is blocking Ca^{2+} mobilization or inactivating PLA_2 in the macrophage, we would expect an inhibition of the release of AA, and a consequent inhibition of PGE_2, a result that was observed in our studies.

EFFECT OF MANOALIDE ON PAIN AND EICOSANOID RELEASE IN AN *IN VIVO* MODEL

Initial pharmacological evaluation of MLD revealed potent antagonism of PMA-induced local inflammation in the mouse epidermis, and inhibition of phenylquinone writhing (De Freitas et al. 1984). Eicosanoids are released when mouse skin is exposed to diverse stimuli (Carlson et al. 1985; Opas et al. 1985). Intraperitoneal injection of zymosan will induce writhing and the synthesis of LTC_4 and PGE_2 (Doherty et al. 1985) and 6-keto-PGF1α in mice (Doherty et al. 1987). Since we showed that MLD inhibited both PGE_2 and LTC_4 production in cultured mouse peritoneal macrophages, we decided to investigate if the analgesic effect of MLD on zymosan-induced writhing in the mouse was correlated with a reduction in the levels of both LTC_4 and 6KPGF in mouse peritoneal exudates.

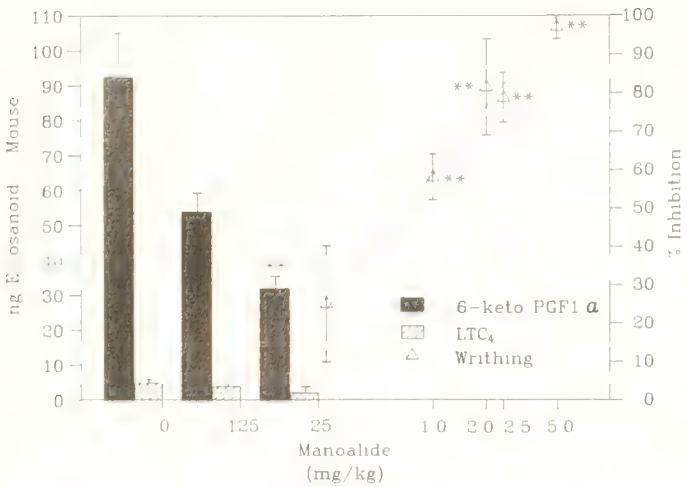

FIGURE 8. Effect of MLD on peritoneal writhing and release of LTC_4 and 6KPGF in mice injected with zymosan (1 mg) i.p. MLD inhibited peritoneal writhing (ED_{50} = 0.71 mg/kg) and the release of LTC_4 (ED_{50} = 0.24 mg/kg) and 6KPGF (ED_{50} = 0.20 mg/kg). Reproduced from Mayer et al. 1988 with permission of Waverly Press Inc

MLD treatment produced a dose-dependent inhibition of zymosan-induced writhing and inhibition of release of 6KPGF (Fig. 8). In contrast to the observed zymosan stimulated increase in LTC_4 production *in vitro*, LTC_4 release was dose-dependently inhibited *in vivo* (Fig. 8). Furthermore, the ED_{50} for both LTC_4 and 6KPGF corresponded to only a 25% reduction of peritoneal writhing, thus suggesting that the analgesic effect of MLD in zymosan-stimulated peritoneal writhing is only partially correlated to the inhibition of eicosanoid production. Zymosan-induced writhing is a complex biological response in which eicosanoids and other mediators such as complement may also intervene (Jose et al. 1983).

FUTURE PERSPECTIVES

In conclusion, our research with MLD up to the present time has suggested the following:

1) Naturally occurring inhibitors of PLA_2, with the potential to regulate phospholipid metabolism in mammalian systems as shown in our studies, are present in marine sponges and may be discovered in other marine invertebrates. It remains to be investigated what the normal physiological role of these compounds is in these organisms. One way to approach this question may involve culturing the *Luffariella variabilis* sponge under defined conditions where eicosanoid metabolism and MLD production can be closely monitored.

2) MLD is a potent inhibitor of PLA_2. The mechanism of inactivation is irreversible and extremely potent. Recent studies have suggested that the MLD binding site on the enzyme may correspond to the substrate binding site. Future studies will address this question and focus on the precise characterization of the interaction of MLD with its binding site on the PLA_2 enzyme.

3) Our studies on the mode of action of MLD have clearly demonstrated that MLD affects the release of AA from mouse peritoneal macrophage membrane phospholipids, and that this in turn affects eicosanoid release when these cells are stimulated with various agonists. It is clear to us that MLD may become a useful drug for the elucidation of biochemical pathways involved in phospholipid breakdown and metabolism in these and other cell types. Work is currently underway to clarify the mechanisms involved in AA release in agonist-treated macrophages in the presence of MLD.

4) MLD has a potent *in vivo* effect on the production of eicosanoids in mice, thus suggesting that this drug may be useful in treatment of disorders where eicosanoids have been shown to participate. MLD is currently being investigated as a candidate drug for the treatment of skin diseases where activation of phospholipases and the presence of eicosanoids has been documented. The successful development of MLD for clinical use may pave the way for the future use of this drug in other clinically relevant syndromes.

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New Pharmaceuticals from Cultured Blue-Green Algae

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INTRODUCTION

The pharmaceutical industry has witnessed explosive growth in the last fifty years, primarily due to the discovery and development of antibiotics and other drugs from fermented microorganisms. As a consequence, bacterial infection is no longer the prime killer that it was in the first quarter of this century. Cancer and heart disease are now the leading causes of death in the United States and acquired immune deficiency syndrome (AIDS) looms as a potential major killer.

Effective drugs for the treatment of these latter diseases still need to be found. To reach this objective the pharmaceutical industry continues to search for new drugs from traditional sources such as fermentation products. In the last decade, however, the discovery rate of new drugs has decreased to a point where certain drug companies no longer find it profitable to continue support of their fermentation products programs. As a result, these drug companies (e.g., Hofmann-La Roche and Smith Kline-Beckmann) have terminated them.

Microalgae represent a source of cultivable microorganisms that have been essentially unexploited for their pharmaceutical potential, both by industry and academia. Prior to initiation of work on other secondary metabolites of blue-green algae (cyanobacteria) in the mid 1970s, anatoxin-a, a potent neurotoxin from the freshwater, filamentous cyanophyte *Anabaena flos-aquae,* was the only natural product from a blue-green alga that had been identified structurally (Huber 1972; Devlin et al. 1977). In the last decade, however, over one hundred unusual natural products from blue-green algae have been described (Moore 1981; Faulkner 1984, 1986). Interestingly, the pharmacological data that have been accumulated to date strongly suggest that the discovery rate of new, useful drugs from this phylum of prokaryotic organisms may be comparable to that from bacteria and fungi.

PROBLEMS ASSOCIATED WITH FIELD-COLLECTED MATERIAL

Relatively few active species of blue-green algae can be collected in the field in large enough quantities for extensive pharmacological evaluation and development. Among the most abundant species are toxic strains that frequently grow in eutrophic freshwater lakes and reservoirs. *Microcystis aeruginosa,* a colonial blue-green alga that produces several hepatotoxic cyclic heptapeptides called microcystins or cyanoginosins (Botes et al. 1984), is the most common cyanophyte found in freshwater blooms. This blue-green alga has been responsible for significant losses of livestock that have drunk the water from lakes and ponds in which toxic blooms have suddenly appeared (Carmichael 1986; Galey et al. 1987). *Anabaena flos-aquae,* which produces neurotoxic alkaloids called anatoxins, is the second most common species of toxic blue-green alga associated with freshwater blooms (Carmichael 1988). This alga has also been involved in animal kills (Mahmood et al. 1988). *Nodularia spumigena,* another filamentous, hepatotoxic cyanophyte that has been implicated in the mortality of wild and domestic animals, grows abundantly in brackish-water lakes and estuaries, including the Baltic Sea (Edler et al. 1985). *Nodularia spumigena* was the first cyanophyte to be reported as toxic (Francis 1878). All of these organisms grow so well under eutrophic conditions that there is concern about the health hazard they pose to drinking and recreational water supplies.

In the marine environment massive blooms of planktonic blue-green algae are less common. Species of *Trichodesmium* can grow abundantly in tropical seas where water temperature is above 25°C and the salinity is near 35‰. *Trichodesmium* blooms in general are non-toxic, although Sato et al. (1963) suggested that they may be responsible for human disease, and Ferguson Wood (1965) pointed out that they are sometimes inimical to fish. Nothing is known about the pharmacological activity of *Trichodesmium,* although Ramamurthy (1970) suggested that *T. erythraeum* may be responsible for antibacterial activity detected in the intestinal tracts of pelagic fishes.

Lyngbya majuscula is one of the most abundant benthic cyanophytes in the ocean. This filamentous cyanophyte is a major seaweed on the reefs of Oahu, Hawaii, and is responsible for a severe contact dermatitis that sometimes affects swimmers during the summer months on the windward side of the island (Moore 1982). Another variety of *L. majuscula* that grows abundantly on the coral pinnacles in the lagoon of Enewetak Atoll contains a potent fungicide, majusculamide C, which is active against several fungal plant pathogens (e.g., *Phytophthora infestans,* the causative organism of tomato late blight, *Plasmopora viticola,* the causative organism of grape downy mildew, and *Rhizoctonia solani,* the causative organism of Rhizoctonia damping-off), including strains that are resistant to at least one fungicide currently on the market (Carter et al. 1984). This cyclic nonadepsipeptide is the first natural product with potential commercial value from a blue-green alga. Unfortunately, this variety of *L. majuscula* has been found only in deep water at Enewetak and consequently adequate amounts of majusculamide C are unavailable for advanced testing and development.

In summary, research and development of pharmaceuticals from blue-green algae using field-collected material is hindered by the unavailability of a large number of active species in adequate biomass, and the frequent inaccessibility of those few species that do grow in large quantity.

A more serious problem than quantity is quality of the drug-producing cyanophyte found in the field. Generally secondary metabolite production is very unpredictable since it is influenced by a variety of environmental factors. Season, for example, plays a major role. In South Africa, *Microcystis aeruginosa* grows abundantly in many freshwater lakes and reservoirs all year around, but only during the summer months is it toxic (The Limnology of Hartbeespoort Dam 1985). Periodicity is another

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important factor. Secondary metabolite production can vary tremendously within a bloom. For instance, hydrophilic extracts of a marine *Phormidium* species from Pohakuloa on the island of Molokai, Hawaii, and an *Oscillatoria* species from Arumizu Bay, Palau, both showed good anticancer activity (Moore 1982), but unfortunately neither organism proved to be active on recollection. Habitat also affects secondary metabolite production. For example, the *Lyngbya majuscula* which grows at Kahala Beach, Oahu, produces an indole alkaloid, lyngbyatoxin A, but the varieties found at adjacent beaches such as Diamond Head do not. Majusculamide C is found in the *L. majuscula* growing at depths of 15–30 m in Enewetak lagoon, but not in the *L. majuscula* growing in shallow water (9 m) in the lagoon. Undoubtedly genetic factors also contribute to some of the variability seen in secondary metabolite production in the field.

ADVANTAGES OF CULTURING

Potentially, any blue-green alga can be grown in mass culture. In contrast to a few active species that are always abundant in the field, many active species can be made abundant through cultivation. Cultivation is the only means whereby drug production can be studied in rare species or cyanophytes that never grow to substantial levels in the field.

Whereas drug production can vary markedly in field samples, yields can be stabilized in culture by controlling conditions. Once growth conditions have been optimized for drug production, the cultured organism provides a continuous source of material. Since drug production and cell growth can vary widely between cells isolated from the same colony, culturing allows cloning of high drug-producing strains to improve yield.

Laboratory cultures can be manipulated for chemical studies that are impossible with the wild organisms, such as the isolation and identification of extracellular metabolites and biosynthetic studies. Hirosawa and Wolk (1979), for example, were able to isolate and characterize an extracellular substance from the phosphate-free culture medium of *Cylindrospermum licheniforme* that stimulated akinete formation in the cyanophyte. Certain biological studies can also be done with the cultured organisms that are impossible with those found in the field. For example, laboratory cultures of algal symbionts in certain drug-producing marine sponges and tunicates can be prepared to evaluate the role of the symbiosis in drug production. Block mutants can be produced for studies on biosynthesis.

Of greater importance, genetic manipulation of blue-green algae in laboratory culture is possible, providing a means to increase production of the most interesting natural products. Gene shuttle systems are becoming available for both genetic analysis and introduction of engineered genes.

PROBLEMS WITH CULTURING

The biggest problem with culturing blue-green algae for drug production is the relatively high cost. It is, at present, much more expensive to culture a blue-green alga than to culture a bacterium or a fungus for a specialty chemical. One reason is that most blue-green algae grow almost an order of magnitude more slowly than do bacteria and fungi. Second, drug yields from cultured blue-green algae are frequently lower than drug yields from bacteria and fungi. In addition, yields from cultured blue-green algae are frequently lower than yields from most field collections of blue-green algae.

The slow growth rates hinder not only the availability of drugs, but make manipulation of the organism for laboratory studies (e.g., optimization and mutation) much slower and more difficult than with bacteria and fungi. Great care must be taken in preparing inocula, media, and vessels for production cultures, as traces of rapid-growing contaminants can quickly dominate the cultures.

Since the vast majority of blue-green algae are obligate photoautotrophs, ultimate bioreactor size for mass cultivation is limited by availability of light, a factor which is not significant in heterotrophic (e.g., bacteria and fungi) fermentations.

Frequent loss, alteration, or decrease of desired drug production in the cultured alga is a serious problem. Secondary metabolite production is often very sensitive to environmental factors. Therefore, substantial effort and time must be expended to determine chemical and physical conditions appropriate for optimum growth and drug production. Even when seemingly stable strains of drug-producing cyanophytes are obtained using conventional cloning techniques, drug production sometimes disappears on repeated subculturing, especially if culture conditions are modified. For example, anatoxin-a toxicity in *A. flos-aquae* NRC-44-1 disappeared when the medium was changed from ASM-1 to the nitrate-richer BG-11. Similarly, repeated subculturing of *An. flos-aquae* S-23-g, a strain that produces the neurotoxin anatoxin-d, on ASM-1 followed by BG-11 resulted in loss of neurotoxicity and expression of hepatotoxicity similar to that observed in *Microcystis* (Carmichael 1986).

Spontaneous mutation can result in loss of bioactive stock cultures, especially ones maintained on agar slants for extended periods of time. There is no guarantee, however, that drug activity will be preserved in frozen or lyophilized stocks, since pharmacological activity is frequently lost on reconstitution.

The complete removal of contaminants, especially adherent bacteria, presents one of the biggest difficulties in preparing pure cultures of cyanophytes. The preparation of a bacteria-free, or axenic, culture of the drug-producing blue-green alga is desirable for at least two reasons. First, axenic culture unambiguously establishes the origin of the drug activity. Second, an axenic culture is necessary for studies on biosynthesis of the drug, especially those involving feeding of substrates (e.g., labeled acetate, sugars, and amino acids) that can be readily metabolized by bacterial contaminants as well as by the blue-green alga.

Axenic cultures can be prepared from unialgal cultures by carefully selecting uncontaminated growing cells, and replating at frequent intervals. Blue-green algae that have heavy extracellular sheaths are generally difficult to obtain in axenic culture. Bacterial contaminants are found predominantly in and on the sheath. Rapid-growing planktonic blue-green algae, however, frequently lose their sheaths in culture, which makes bacteria-free cultures easier to obtain (Carmichael and Gorham 1974). Soil cyanophytes, which are more likely to have lower growth rates and tend to retain their sheaths in culture, are therefore much more difficult than planktonic cyanophytes to obtain in axenic culture. Antibiotic procedures, which are commonly used for purifying eukaryotic cultures, work poorly or not at all with prokaryotic blue-green algae.

PROTOCOL

Collection and acquisition of samples

The algal culture program at the University of Hawaii was initiated in 1981. From the onset the program was not restricted to the cultivation of marine blue-green algae, as there was no reason to believe that new pharmaceuticals could not be found in freshwater and terrestrial species. Moreover it was strongly suspected that some freshwater and terrestrial cyanophytes might be producing secondary metabolites similar to those found in marine species, since, like bacteria, many species of cyanophytes can adjust to a diversity of growth conditions and habitats.

Over 500 strains of blue-green algae had been grown in mass culture and evaluated for pharmacological activity by mid-1986 (unpublished reports). All had been isolated from field samples collected in a variety of freshwater, terrestrial and marine environments, mostly soils and other terrestrial habitats such as the surfaces of rocks and buildings. In September 1986 work began to culture an additional 1,000 strains of blue-green algae over a five-year period to provide the National Cancer Institute (NCI) with extracts for selective cytotoxicity testing against a panel of 100 human cancer cell lines and for evaluation against AIDS. In addition to isolates from field-collected samples, strains from culture collections such as the American Type Culture Collection (ATCC) and the University of Texas Culture Collection (UTEX) are being grown in batch culture for the NCI contract work.

Taxonomic identification

Classification of cyanophytes has, for over 200 years, depended on gross morphology of the algal cells, in particular the reproductive bodies (spores, hormogonia and akinetes) and specialized cells (heterocysts), as well as the morphology of the colloidal extracellular material known as the sheath that accumulates around the algal cells. Many morphological features found in wild material, which are important for both generic and specific identification, disappear when the alga is grown in culture, such as the thick sheath of mucopolysaccharide that surrounds the algal cells of *Microcystis aeruginosa*. Thus, examination of field-collected material is necessary for identification of a blue-green alga that has been brought into culture.

Many classical taxonomists placed emphasis on morphology of the sheath, failing to recognize its variability in response to habitat. As a consequence, named taxa have proliferated to such an extent that many phycologists question the merit of the classical system as described by Geitler (1932), Fritsch (1942, 1945) and Desikachary (1959). The revised system of Drouet (1968, 1973, 1978, 1981; Drouet and Daily 1956), which concentrates on the morphology of the algal cells, has greatly reduced the number of named taxa, but many phycologists feel that the system is oversimplified. Recently, the International Association for Cyanophyte Research has recommended that use of Drouet's system be abandoned (Golubic et al. 1985). To further complicate matters, a unilateral proposal (Stanier et al. 1978) to place the classification of the blue-green algae under the rules of the International Code of Nomenclature of Bacteria (ICNB), coupled with an associated movement to regard the blue-green algae as bacteria, has resulted in the classification of the blue-green algae according to two different codes. Microbiologists now refer to the blue-green algae as cyanobacteria under the ICNB, but botanists include blue-green algae within the scope of the Botanical Code and will continue to do so in the future (Golubic et al. 1985). This means that conscientious researchers, especially those who isolate previously uncultivated organisms from the field, must categorize their isolates according to three different taxonomic schemes.

Preliminary screening

For screening programs that have a narrow scope or ones that use relatively simple bioassays requiring only a small amount of extract for testing (for example, *in vivo* assays such as lethal toxicity or ear skin inflammation in the mouse and *in vitro* assays such as cytotoxicity, and antimicrobial and antiviral activity), preliminary screening of field-collected material can focus the researcher's attention on which organisms to isolate and mass cultivate. Since activity observed in field-collected material is frequently altered or lost in culture, knowledge that an interesting activity exists in the field-collected sample alerts the investigator that the alga should be grown under a variety of conditions to maximize chances of the desired activity being expressed in culture.

Isolation of algal strains

Generally, samples collected in the field for the University of Hawaii program are small amounts of soil or algal matter that are transported to the laboratory in sterile disposable polyethylene bags (Whirl-Paks). Each sample is first dispersed in liquid culture medium that contains nitrate as the sole nitrogen source, and no organic carbon source (Carmichael 1985). This favors the growth of blue-green algae while retarding growth of other microorganisms. Aliquots of the dispersed sample are then used as inocula to prepare cultures for isolation using the direct isolation technique, either alone or in conjunction with a stage of culture enrichment prior to direct isolation.

Direct isolation involves dispersal of the organisms in the inoculum directly onto or into a selective solid medium. The selective medium is usually one of the mineral-based liquid media described in the literature for blue-green algae, e.g., Allen's medium 3 (1952), which has been solidified with agar. Colonies derived from a single cell or a short fragment of a filament are selected for isolation and aseptically transferred to the surface of a fresh agar plate. This procedure is repeated successively until unialgal cultures are obtained.

The enrichment technique is particularly useful for samples containing fungi and other microorganisms that might rapidly dominate a direct isolation culture. For enrichment of blue-green algae from soil or water samples, the dispersed sample is grown in a selective liquid medium supplemented with certain growth inhibitors of contaminants. For example, cycloheximide can suppress growth of fungi, and germanium dioxide can inhibit growth of diatoms. After 2–3 weeks of incubation under low light, a portion of the algal growth is removed, homogenized, and plated as described for the direct isolation procedure.

Axenic cultures may, in some cases, be prepared from unialgal cultures by carefully selecting uncontaminated, growing cells

and transferring them aseptically and frequently to fresh plates. For groups of blue-green algae that cannot be purified by this method, alternative procedures are used. Individual cells or short fragments of filaments, which lack an extracellular sheath and are large enough to be seen under the dissecting microscope, may be purified by mechanically removing the cell from the surrounding matrix and capturing it in a narrow capillary pipette, after which it may be transferred through sequential washings in relatively large volumes of sterile medium to eliminate smaller contaminants (Hoshaw and Rosowski 1973). Fragments of filaments of planktonic forms may be purified by passing a suspension of the alga through a membrane filter that retains the filaments while allowing passage of contaminating bacteria (Heaney and Jaworski 1977). Members of the Oscillatoriaceae, which are capable of gliding motility, may be purified by repeatedly cutting away and transferring portions of the filament near the advancing edge of the migrating population. Contaminating organisms are left behind, immobilized on the plate (Bowyer and Skerman 1968). Non-motile filamentous blue-green algae, especially those with heavy extracellular sheaths that frequently harbor a wide variety of contaminants, can be mechanically disrupted by short-term blending or sonication, either with or without detergents to aid in dissolution of the sheath (Brown and Bischoff 1962; McDaniel et al. 1962). After disruption, fragments of filaments free of sheath and adherent bacteria can be carefully selected and plated. The preparation of some bacteria-free cultures has taken advantage of the apparent greater resistance of blue-green algae to increased temperature (Kratz and Myers 1955; Allen and Stanier 1968) or to ultraviolet irradiation (Gerloff et al. 1950). Although antibiotic procedures generally work poorly or not at all with blue-green algae, some reduction in bacterial populations may be achieved by dark incubation in the presence of antibiotics and a medium that favors bacterial growth (Vance 1966; Lorenz and Krumbein 1984).

Stock cultures are maintained on agar slants at room temperature under low light intensity. Preservation of the blue-green algae for long periods of time is achieved by storing both frozen stocks and lyophilized stocks in the vapor phase of liquid nitrogen.

Production cultures

When the culture progam was initiated at the University of Hawaii in 1981, it was decided that selection of algal isolates from field samples or culture collections for production cultures would not be biased by preliminary screening data, since pharmacological and agrochemical evaluation of extracts of the cultured algae would not be limited to the few assays mentioned above. Organisms would be chosen to provide the program with a broad taxonomic representation of the families as well as geographical and habitat ranges of blue-green algae. Each isolate would be grown in production cultures using one set of growth conditions. Modification of the growth conditions (e.g., pH, culture medium) would be made, however, as recommended by the literature, to favor the growth of a particular family or genus. Using this approach, several field-active cyanophytes were expected to lose their activities when brought into culture. In actuality this has appeared to be the case. For example, in work carried out in the late 1970s and early 1980s, cytotoxicity (also *in vivo* antineoplastic activity) had been detected in >15% of the extracts of field-collected blue-green algae (unpublished results). The percentage of extracts of cultured blue-green algae showing cytotoxicity, however, has been significantly lower (6%).

Preparation of production cultures of all algal isolates is serving two important purposes. Sufficient extract of each alga can be obtained from production cultures for 1) submission to various in-house, academic, industrial, and government laboratories for evaluation in existing assays, and 2) placement in a repository where the extract will be available for evaluation in new assays as they are developed.

For production cultures, each algal isolate is tested in a variety of growth media to determine which of the various standard formulations yields acceptable growth. The cultures are then sequentially scaled up in volume until sufficient material is available to inoculate 10 L production cultures. Incubation of the production cultures is carried out under the following conditions: continuous illumination at an incident intensity of 100–350 microEinsteins m^{-2} sec^{-1} from mixed banks of cool-white and warm-white fluorescent tubes; aeration with sterile air containing 0.5% carbon dioxide at a flow rate of 0.1 to 0.2 volumes of gas per volume of culture per minute; and a temperature of 24 ± 1°C. Cell growth parameters are monitored daily and the production cultures are harvested in the early portion of the stationary phase of growth, typically 15 to 25 days after inoculation.

The majority of filamentous blue-green algae that form large clumps are separated from the culture medium by filtration. For many unicellular forms and some filamentous cyanophytes, especially planktonic ones, cells are separated from the culture medium by a refrigerated centrifuge equipped with a continuous-flow attachment, or by tangential flow membrane filtration. The harvested cells are then rapidly frozen and lyophilized. Yields of lyophilized cells range from 0.1 to 1.0 g/L of culture. The lyophilized cells are stored at −20°C prior to extraction.

Extraction and pharmacological evaluation of extracts

Extracts of lyophilized cells are prepared for pharmacological evaluation by first treating the cells with 3:7 ethanol : water and then with 1:1 dichloromethane : 2-propanol. The amounts of hydrophilic and lipophilic extract vary substantially from alga to alga.

Each hydrophilic extract and each lipophilic extract is tested for cytotoxicity against the KB cell line (a human nasopharyngeal carcinoma), antifungal activity against five test organisms, antibacterial activity against 12 test organisms, and antiviral activity against *Herpes simplex* type II virus and respiratory syncytial virus. In addition, extracts are screened for a wide variety of other pharmacological activities such as tumor-promoting and anti-tumor-promoting activity, antiinflammatory activity, cardiotonic activity, central nervous system (CNS) activity, and immunomodulating activity. Some of the latter testing is being carried out at the University of Hawaii, but most of the work is done elsewhere, primarily in industry.

When an interesting activity is discovered, a second 10 L production culture is prepared and the hydrophilic and lipophilic extracts of the new batch are tested for reproducibility of

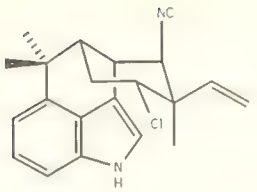
the activity. Some of the organisms, for unknown reasons, fail to show activity on regrowth. For those blue-green algae showing reproducible activity, production cultures are scaled up to 25 L to provide adequate material for isolation and identification of active compounds and up to 175 L to provide for advanced pharmacology.

Extraction of lyophilized cells for biotoxins is different. Biotoxins, which are generally more water soluble than most other secondary metabolites in blue-green algae, are extracted from cells using water alone or mixed with methanol, ethanol, or butanol. Hydrophilic extracts are monitored for bioactivity throughout the extraction procedure by using small animal assays (i.e., mouse lethality by intraperitoneal injection). The various toxins can be monitored by their different signs of poisoning (i.e., nerve or organ toxicity). Preliminary screening of field material for toxicity, prior to isolation of strains, can be accomplished by intraperitoneal injection of freeze-dried or freeze-thawed cells. Large scale production of biotoxins for structure/function studies requires multiliter quantities as do the other bioactive chemical-producing strains.

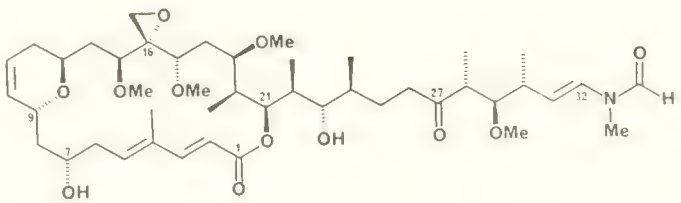
Optimization of drug production

Very little work has been done at the University of Hawaii or elsewhere on selecting strains and determining optimum culture conditions for maximum drug or toxin production. To date, optimization studies have been carried out at the University of Hawaii on only one *Oscillatoria acutissima*. This freshwater species produces two cytotoxic macrolides called acutiphycins, both of which show antineoplastic activity against Lewis lung carcinoma in mice. The studies indicate that concentrations of major nutrients and harvest time are very important for modulating acutiphycin production. Drug yield is highest when nitrate and phosphate are present in limiting or near-limiting amounts. The amount of acutiphycin is drastically reduced when the concentration of either nutrient is increased. Addition of organic carbon or nitrogen to the mineral medium likewise decreases acutiphycin production. Acutiphycin yield is at a maximum when the alga is harvested after three weeks, at which point the culture is in a stationary phase of growth. A lower yield is obtained if harvest is in the late stages of the stationary phase. Concurrent with a decrease of acutiphycin production in the later stages of the stationary phase is an increase in production of extracellular colloids which interferes with drug isolation. This pattern of varying secondary metabolite production with growth conditions is also seen with biotoxin production by planktonic cyanophytes (Carmichael 1986).

Optimal culture conditions are achieved by varying the environment surrounding the growing cell and observing the effects on growth and drug production. The optimization process requires a large number of experiments and is consequently very time-consuming. Each experiment is generally carried out in triplicate and each culture is assayed, either for bioactivity or for drug yield using HPLC or some other suitable method of analysis. The parameters that are varied are, in approximate order of priority, incubation period, pH, aeration rate, temperature, illumination intensity, mineral nutrition, photoperiod, carbon and nitrogen sources, presence of contaminating microorganisms, and vessel size and configuration.



hapalindole A



scytophycin B

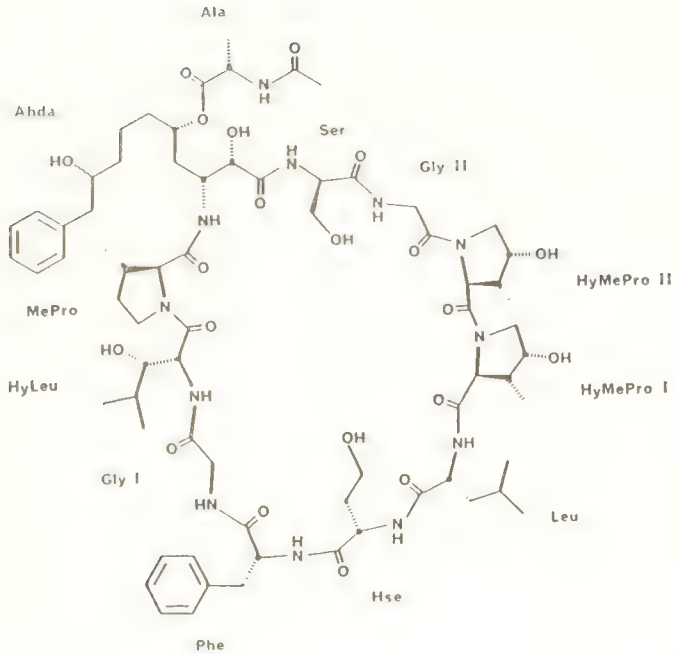
Biosynthesis and genetic studies

Secondary metabolite production from cultured blue-green algae is not very economical. If useful and commercially-important drugs are going to be developed from this new source, methods will have to be found to increase drug production. It is doubtful that many blue-green algae will be grown in mass culture at low cost. Genetic engineering and recombinant DNA technology (Craig and Reichelt 1986; Porter 1986) will probably be very useful tools for helping to solve production problems.

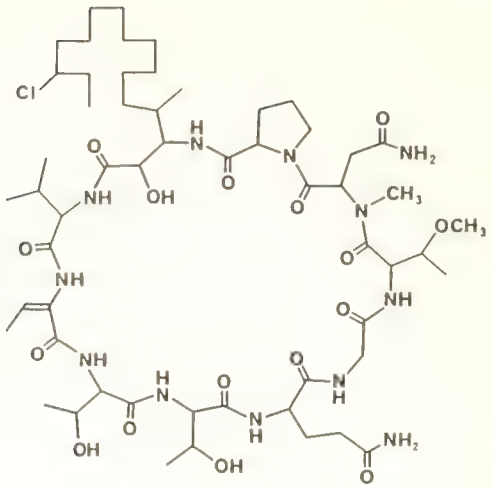
Basic studies on the biosynthesis of secondary metabolites found in blue-green algae are needed first. The only meaningful study of biosynthesis in the literature to date deals with the biosynthesis of saxitoxin in *Aphanizomenon flos-aquae* (see Shimizu et al. 1984; Shimizu 1986). At the University of Hawaii, studies on the biosyntheses of several secondary metabolites of blue-green algae have been initiated.

Although gene cloning systems have been developed for some unicellular (*Anacystis* and *Agmenellum*) (Porter 1986) and filamentous (*Anabaena, Nostoc,* and *Plectonema*) (Wolk et al. 1984; Flores and Wolk 1985) cyanophytes, no studies of the genetics of secondary metabolite production have been carried out yet. Methods still need to be developed to clone genes that are involved in secondary metabolite production. Plasmids are found in several pharmacologically active cyanophytes and therefore it should be possible to use them to clone genes.

Interestingly, plasmids may be involved in the production of the toxins in *Microcystis,* but not in the production of the toxins in *Anabaena.* Hauman (1981) found that the toxicity of *M. aeruginosa* WR70 is completely lost when this strain is treated with a relatively low dose of acridine orange, an agent that



scytonemin A



puwainaphycin C

interferes with plasmid replication but not with chromosomal replication. Kumar and Gorham (1975), on the other hand, found that the toxicity of *An. flos-aquae* NRC-44-1 is not lost after treatment of the strain with acridine dyes.

RESULTS

The blue-green algae appear to be excellent sources for new cytotoxins and fungicides. Six percent of the extracts of the cultured algae show cytotoxicity against the KB cell line with MICs <30 μg/ml and 9% of the extracts show antifungal activity at 1 mg/disc against one or more test organisms, viz. *Aspergillus oryzae, Candida albicans, Penicillium notatum, Saccharomyces cerevisiae,* and *Trichophyton mentagrophytes.*

To date, several new cytotoxins and antifungal agents have been isolated and identified in our laboratory. The acutiphycins from *Oscillatoria acutissima,* for example, are novel macrolides that show cytotoxicity against KB at <1 μg/ml and activity against murine, intraperitoneally implanted Lewis lung carcinoma with T/C = >150 at 50 mg/kg (Barchi et al. 1984). The hapalindoles from *Hapalosiphon fontinalis* are novel indole alkaloids, some of which are isonitriles and others isothiocyanates (Moore et al. 1987). The isonitrile-containing hapalindoles show moderately-strong antifungal activity against *C. albicans, T. mentagrophytes, Neurospora crassa,* and *Saccharomyces pastorianus.*

The scytophycins from *Scytonema pseudohofmanni* are unusual macrolides that show cytotoxicity against KB at 1 ng/ml and moderate activity against murine, intraperitoneally implanted P388 lymphocytic leukemia and Lewis lung carcinoma with T/C = 130 at 0.2 mg/kg (Moore et al. 1986). Tolytoxin from *Tolypothrix conglutinata* var. *colorata* and *Scytonema mirabile* is a macrolide that is structurally related to the scytophycins and shows essentially the same cytoxicity against KB as do the scytophycins. Tolytoxin and all of the scytophycins are also potent broad-spectrum fungicides that have molecular structures and biological activities comparable with those of the swinholides, ulapualides, and kabiramides. The latter three groups of compounds are marine natural products which have been isolated from certain sponges, nudibranchs that feed on the sponges, and the egg masses of these nudibranchs. Interestingly, the sponges that elaborate these compounds contain algal symbionts, which suggests that the symbionts may play a role in the biosynthesis of these highly bioactive macrolides.

A large number of extracts show antibacterial activity, but the compounds that have been isolated so far exhibit only weak activity. Scytonemin A from *Scytonema* sp. U-3-3, for example, shows weak activity against both Gram-positive and Gram-negative bacteria. This novel cyclic peptide, however, is a moderately strong calcium antagonist (Helms et al. 1987).

A fairly large percentage of the hydrophilic extracts show cardiotonic activity in isolated mouse atria. An increase in chronotropic activity has been attributed in several cases to tyramine (unpublished results). In four species of blue-green algae belonging to the Scytonemataceae, positive inotropic activity has been linked to a class of compounds of unknown structure called the tolypophycins (unpublished results). In another blue-green alga, tentatively identified as an *Anabaena* sp., positive inotropic activity has been associated with an unusual chlorine-containing cyclic peptide, puwainaphycin C (Gregson 1986).

Blue-green algae appear to be an excellent source for new antiviral agents (Rinehart et al. 1981). Over 5% of the extracts of cultured blue-green algae show antiviral activity against *Herpes simplex* virus type II and another $>5\%$ show activity against respiratory syncytial virus (unpublished results). To date, however, none of the active compounds have been isolated and identified.

Almost all of the pharmacologically-active compounds that have been isolated from blue-green algae to date have unique structures. In only two instances have known drugs been isolated, viz. tubercidin from *Tolypothrix byssoidea* (see Barchi et al. 1983) and toyocamycin from *Tolypothrix tenuis* (Stewart et al., in press).

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National Cancer Institute's Role in the Discovery of New Antineoplastic Agents

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INTRODUCTION

The objectives of this paper are to give an overview of the status of the U.S. National Cancer Institute's (NCI) drug development program with particular reference to natural products and even more specifically towards marine natural products. We wish to present the philosophies, approaches, objectives, and current and future directions of the NCI program as an example to illustrate the kinds of considerations involved in drug development and the processes a newly isolated marine compound would have to go through to reach clinical trials. This program is designed specifically to discover substances from marine organisms with selective effects against slow-growing solid tumors such as lung and colon cancers. We conclude with a discussion of the program for collection and screening of marine macroorganisms that we have established, and discuss special considerations in recollection, scale-up, and advanced development that are pertinent to marine organisms.

There are several approaches to cancer therapy, including surgery, radiation therapy, and chemotherapy, but this paper will deal with only one area of chemotherapy, the discovery and development of new direct-acting antitumor agents. Other areas of chemotherapy that are quite important, but which will not be further discussed here, are agents that modulate response to radiotherapy by either sensitizing the hypoxic cancer cells to radiation (radioenhancers) or protecting normal tissues from radiation (radioprotectors), and agents that modulate the immune system to enhance activity of lymphocytes and macrophages.

OVERVIEW OF DRUG DISCOVERY AND DEVELOPMENT

There are two major avenues of approach to drug discovery, the so-called "rational" and "empirical" routes. The former bases its conceptual approach on investigation of the molecular basis of disease and, once having discovered the biochemical abnormality at the cellular level, uses that information to establish the three-dimensional structure of the key molecules (biological targets or receptors) and then designs antagonists to inhibit the abnormal process. This type of approach has certainly had impressive therapeutic successes but it is a slow, step-by-step process, depending on fundamental scientific breakthroughs and the ability to exploit them in a coordinated way.

The NCI program has tried to improve the efficiency of this process by setting up a major research grant process of cooperative agreements among the NCI, basic scientists in molecular biology, chemistry and biochemistry, and pharmaceutical interests to form National Cooperative Drug Discovery Groups (NCDDGs) (see Fig. 1). Each group brings together experts in different aspects of the topic from universities, cancer centers, research institutes, and private companies with the idea of forming working groups to accelerate discovery and passage of key information from one stage to the next. At present four NCDDGs focus on broad mechanisms of cancer that cross tissue or organ groups; they investigate polyamines, topoisomerase, oncogenes, and monoclonal antibodies. There are also two NCDDGs that look for specific biochemistry and molecular biology relating to lung and colon cancer.

The "empirical" approach is to develop models felt to be of predictive use, and then to screen large numbers of substances in those model systems for potentially useful compounds. While some have condemned this approach as a "fishing expedition" and as "second-rate science," to date most useful drugs have been discovered through screening. Thus this approach has much to recommend it. The left half of Figure 1 shows the main areas involved in empirical drug discovery: acquisition of materials to screen, initial high volume *in vitro* screening, and finally more detailed *in vivo* testing of selected active compounds to establish whether they are good candidates for possible development (see also Rinehart's contribution to this volume). All of the new NCI natural product collections will be screened through the "empirical" side of Figure 1.

The "rational" and "empirical" approaches are by no means independent of each other, since basic discoveries of mechanism allow development of new screens for the empirical approach. Likewise, compounds determined active by screens may lead to new basic discoveries when used as tools specifically to perturb molecular and cellular processes. The success of drug discovery clearly depends on both approaches reinforcing each other.

The investigation of natural products as drugs is high risk research since natural products are more expensive and take longer to develop than synthetic ones. The two major areas that cause expense and delay are isolation of pure active compounds and recollection for scale-up. Finding an extract with interesting activity is only the beginning of a process of repeated cycles of chemical separations (fractionation) and bioassay that gradually focus on the active principle(s). This process may take a few weeks to several years depending on the difficulty of the problem. If the active principle is a single entity, is present in substantial quantities (greater than 0.5% of the extract), and is chemically stable, the process goes rapidly. However, when multiple active principles are present in different fractions, are unstable, and/or are in small quantity, a great deal of time is necessary. Also important are reproducibility and speed of the bioassay, and the ability of a chemist to gain access to it. Time waiting in a queue for bioassay at each cycle of fractionation becomes a major delay in isolation of active principles.

Problems with biology, ecology, and abundance of material for recollection can be critical and will be discussed later in this paper, but scale-up isolation nearly always requires much time

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This paper is presented in commemoration of the 100th Anniversary of the National Institutes of Health, 1887–1987.

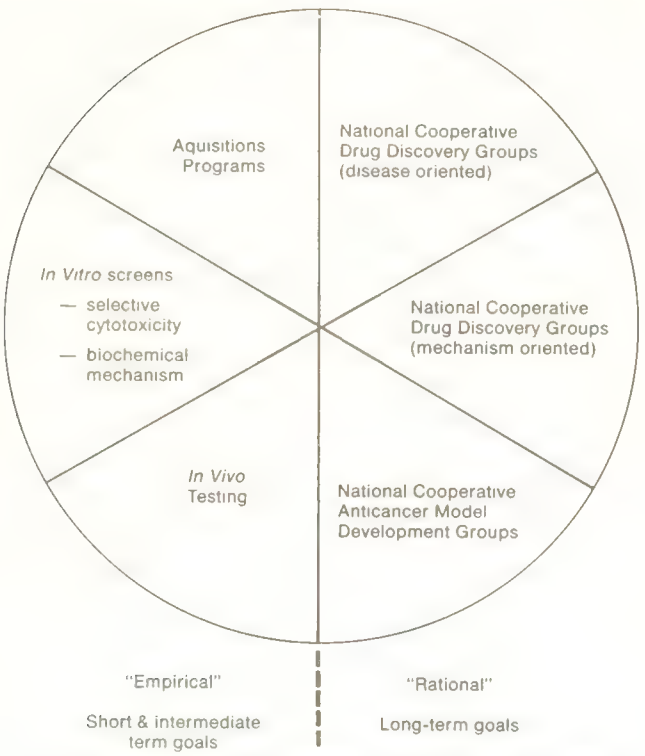

FIGURE 1. National Cancer Institute's anticancer drug discovery program.

and money. The generally low yields of active components (often micrograms to milligrams of pure compound per kilo of starting material) forces use of large volumes of extraction solvents. The complexity of many natural product extracts necessitates multiple chromatographies using large amounts of expensive adsorbents as well. Furthermore, the complexity of separations requires that each process be studied in detail and that special set-ups of pilot plant equipment be tailored to each separation. Hundred gram to kilo quantities are needed for development to clinical studies; high cost of isolating such amounts is an important barrier to development of marine natural products as drugs.

While emphasis on marine natural products as potential drugs has thus far been direct development on a track towards clinical trials, this is not the way most drugs are ultimately developed in the pharmaceutical industry. Figure 2 illustrates three paths from natural products to clinically useful drugs. It is uncommon for the first member of a new class exhibiting a particular pharmacological activity to become a useful drug. In most cases problems such as unacceptable toxicity, inadequate spectrum of activity, insufficient solubility for formulation, or insufficient or costly supply make the direct path (center) impractical to follow. Such leads can be modified (analog development) as on the top path of Figure 2 to increase potency, solubility, and breadth of activity, or to decrease side effects and toxicity.

The longest path, that at the bottom of Figure 2, illustrates a tremendously important aspect of discovery of novel natural

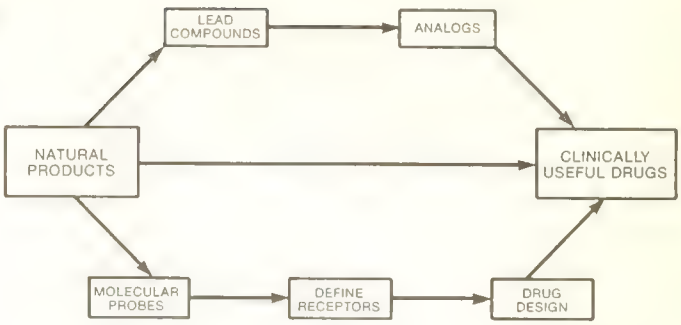

FIGURE 2. Operative pathways in the development of natural products toward clinical trials

products—their use as molecular or biochemical probes. Natural products that are undevelopable because of problems such as those mentioned above may, by detailed mechanistic studies, lead to discovery of a site of action such as a receptor protein, a membrane component, an enzyme, or a fragment of nucleic acid. Even if such sites of attack for inhibition of a key pathway had previously been recognized, there may have been no inhibitors known that specifically affect the site.

Such a molecular or biochemical probe can be developed into a bioassay appropriate for evaluating three categories of compounds: simple analogs, semisynthetic analogs, and congeners. Simple analogs are prepared by easy, high yield reactions that either can add, remove, or chemically modify functional groups on the natural product to yield compounds close to the natural product with differences that may affect their suitability for development. In semisynthesis, a complex natural product is chemically cleaved to yield a simpler nucleus that may be biologically inert but that retains key structural properties; to this nucleus are added fragments to yield derivatives that are not directly accessible from the natural product. The classic example is penicillin: the natural penicillins such as F, G, X, and K, which are minimally useful in modern therapy, are enzymatically cleaved to yield 6-aminopenicillanic acid that is converted into a variety of modern penicillins with highly desirable properties such as broad antimicrobial spectrum, resistance to degradation by *beta*-lactamase, long action, and oral effectiveness.

The third possibility for development on this path is synthesis of congeners, defined in this context as molecules chemically much simpler than the natural product but acting at the same site. The nucleus is generally not the same, but the molecule is designed to have the same overall shape and to have appropriately distributed charge and binding elements so that it binds at the same site as the natural product and does the same job. The classic example of congeners are the synthetic narcotic analgesics such as demerol and dilaudid, which, although in a two-dimensional structural drawing do not resemble morphine, bind very efficiently to the opiate receptor.

Marine natural products will play very important roles as biochemical probes that will lead to useful basic science discoveries. The resultant improved understanding of biochemistry and molecular biology may lead to new drugs as well.

SUCCESSES AND FAILURES IN CANCER CHEMOTHERAPY

Successes in cancer treatment during the last 20 years have been particularly notable in childhood cancers such as leuke-

mias, Wilm's and Ewing's sarcomas, and in such formerly fatal diseases as testicular tumors and Non-Hodgkin's lymphoma. There have really been few new drugs reaching the stage of wide clinical utility in this period, although some, such as dichlorodiamino-*cis*-platinum in testicular cancer, have been important. Rather, the successes have come from the use of combined modalities of treatment and the aggressive use of combination chemotherapy. In the latter, several drugs having different mechanisms of action and different toxicities are combined: this produces summation of effect against tumor cells but not a summation of toxic side effects. The concept was pioneered by Frei and Freireich (1965) with the VAMP regimen (vincristine, methotrexate, mercaptopurine, and prednisone) in acute lymphocytic leukemia, while the MOPP regimen for Hodgkin's disease (cyclophosphamide, vincristine, procarbazine, and prednisone) was introduced by De Vita et al. (1970). The VAMP and MOPP regimens were responsible for the first cures of systemic cancer by chemotherapy.

With few exceptions, adequate chemotherapy for adult solid tumors, such as those of lung, colon, breast, ovary, prostate, pancreas, and brain, is still not available. Furthermore, death rates and incidence of new cases of lung cancer continue to climb at a dramatic rate, increasingly a problem among women as well as men. In 1985, lung cancer passed breast cancer as the leading cause of cancer death in women in the United States, and statistics on lung cancer in men imply that the increase among women is not going to peak or plateau any time soon.

Why has there not been more success in treating solid tumors? Fundamentally, the problem is that existing agents are not very selective for slow-growing tumors: they exert their effects on a largely kinetic basis, with the fastest growing cells taking up more drug and dividing more often, therefore being more sensitive to agents that affect nucleic acid synthesis, mitosis, and other processes associated with growth and division. Compounds highly specific or selective for solid tumors have not been found. One grim possibility is that such compounds do not exist. However, many drugs in a variety of therapeutic areas are tissue- or organ-selective, and many toxins, including those of marine organisms, exhibit considerable specificity. The other main possibilities are that solid tumor selective compounds have not been sought in the right places (which seems unlikely, since a tremendous variety of different synthetic and natural products have been tested) or in the right way with appropriate assays.

SCREENING METHODS—PAST, PRESENT, AND FUTURE

This brings us to analysis of the NCI's experience in development and use of tumor models. The nature of cancer as a slowly developing disease with long onset causes serious problems in modeling. Differences among human tumor types include heterogeneity, growth rate, accessibility, size, and diffuseness, to mention but a few characteristics. From the point of view of chemotherapy, the single most critical difference among human tumors is in drug sensitivity. There is no drug that can be considered broad spectrum, and for many tumor types (e.g., breast) there are marked differences in drug sensitivity among outwardly similar tumors. The positive and negative aspects of many tumor models examined as potential screens since the 1950s have been reviewed elsewhere (Goldin et al. 1966, 1979; Johnson and Goldin 1975; Driscoll 1984; Venditti et al. 1984).

In 1956 the NCI selected L1210 mouse leukemia as a main screen. From 1971 until 1985, P388 lymphocytic leukemia (a somewhat more sensitive system with similar characteristics) served as the primary screen. These screens have done well in detecting compounds with a broad range of activities. Mechanisms of action of compounds highly active in the L1210 and/or P388 leukemia include alkylating agents, purine and pyrimidine antimetabolites, antifolates, mitotic inhibitors, and DNA interactive compounds including intercalators, alkylators, minor groove binders, both single strand and double strand breakers, DNA polymerase inhibitors, topoisomerase inhibitors, and inhibitors of protein synthesis working at a variety of steps in that process.

Both the L1210 and P388 mouse tumors represent rapidly dividing cells, and, in retrospect, the wisdom of those choices might be queried. To understand them requires recognizing the constraints on choosing a screening model. A major consideration of any screen is its throughput—the number of materials that can be tested per unit time with the space, personnel, and funds available. Even with the very large program and resources of the NCI, a throughput of 7,000–12,000 compounds per year necessitated a single primary *in vivo* assay that could be completed in 30 days. To complete an *in vivo* antitumor assay in 30 days requires that tumors have doubling times on the order of 10–15 hours.

Alternatives as primary screens would have been tumors that were more slowly dividing, a battery of tumors having broadly varied doubling times, or *in vitro* assays, all of which were considered unacceptable alternatives at the time. In retrospect, it is not surprising that use of rapidly dividing tumors as a primary screen led to discovery of agents effective primarily against rapidly growing tumors. It is also not surprising that these drugs have very little activity against slow growing tumors in humans.

How can a screening system be designed to look for agents with specificity against lung, colon, and other solid tumors? Use of a single *in vivo* model would reduce throughput dramatically compared to P388 leukemia; use of several slow growing models to look for selective effects (e.g., against lung, colon, breast, or ovarian tumors) would reduce throughput even further, correspondingly reducing the possibility of discovering useful agents. The most logical way to detect agents with high tumor type specificity is to test for selectivity against a wide variety of tumor types. Further, some tissues contain many types of cells that give rise to characteristic subsets of tumors (e.g., lung, Fig. 3); it is known that both distribution and metabolism of drugs in these cell types differ, so each subtype should be included in a screen. Finally, a single tumor from a patient is unlikely to be representative of that tumor in the population as a whole; therefore each subtype should be represented by more than one tumor. Other considerations are that human tumors are preferred to mouse tumors, and that the assays should be highly reproducible and of reasonably short duration. This last point is important not only to throughput, but is also critical to natural products isolation since the bioassay time usually tends to be the rate-limiting step in isolation of active compounds.

Consideration of these requirements led the NCI to the conclusion that the system would have to begin with an *in vitro* screen of modest duration (for throughput), that each tumor type should be represented by several subtypes, and that where

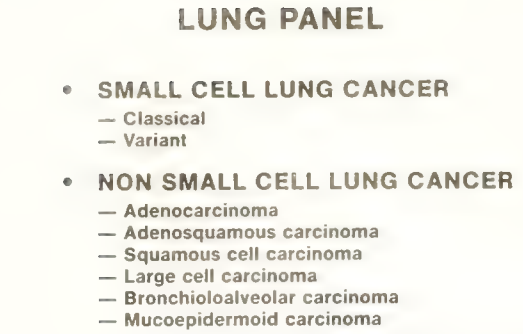

FIGURE 3. Diverse types of lung tumors included in the NCI panel.

possible, each subtype should be further represented by more than one cell line. The system begins with *in vitro* screening to discover selectively active compounds, and follows up with *in vivo* testing using the same tumor lines as xenografts in athymic mice.

The logistical requirements of such an assay system are immense. With 100 cell lines, testing 10,000 samples in each at four concentrations in triplicate requires 12 million (100 × 10,000 × 4 × 3) test cultures, excluding positive, negative, solvent, and media controls. The keys to this type of effort are use of highly automated assays and very small volumes of test samples and media. The basic assay uses a cell viability evaluation with a colorimetric endpoint, modified after Mosmann (1983), done in 96-well microtiter plates. Records of each test substance in the system are maintained by computer, and test scheduling is computer guided. When a technician enters data on which cell lines will be available for testing the following day, the computer determines which samples need to be tested in that cell line, determines which ones should have priority for testing (generally the chronologically oldest samples or the samples that need fewest cell lines to complete testing), and then generates an output diagram for each microtiter plate showing which samples/dilutions are to be placed in each well. This tracks each sample through the entire screen and greatly increases technician efficiency, while reducing errors. Automated plate readers interfaced with computers record optical density at the end of the experiment; these are converted to growth inhibition data. The most time-consuming step at present is sample preparation; the NCI is currently experimenting with use of robots and computer connected balances for weighing initial samples.

The requirements for acceptance of each cell line into the screening panel must be rigorous to assure adequate representation of the human cancers of interest. Acceptability criteria include freedom from adventitious agents (mycoplasma, viruses), karyotypic profiles appropriate to human tissue, tumorigenicity when introduced into athymic mice, and histologic examination of derived tumors. The cell lines should also be from very early passages whenever possible so that they represent the heterogeneity of fresh tumors. Most tumors currently being added to the panel are obtained as fresh isolates through collaborations with surgical cancer groups.

The ultimate goal is for the combined human tumor cell line panels to have 80–100 lines. At present, space limitations restrict this to 50. Large-scale screening and expansion of the screening panels to approximately their final size will occur in 1988 after completion of a dedicated facility for this project. More details of this approach have been published elsewhere (Boyd 1986; Boyd et al. 1988).

TABLE 1. PERCENT ACTIVITY OF MARINE ANIMALS AGAINST P388 IN VIVO MURINE LEUKEMIA IN NCI SCREENS THROUGH 1979.

Group	Species activity (%)	Number tested
Porifera	5.3	1,702
Ctenophora	8.3	12
Cnidaria	4.4	2,089
Annelida	2.5	158
Arthropoda	2.2	1,128
Mollusca	3.7	2,411
Ectoprocta	3.7	190
Echinodermata	3.8	1,515
Tunicata	5.2	425
Chrondrichthyes	3.2	31
Osteichthyes	5.3	2,788
Total	4.3	12,449

There have also been productive developments in establishing corresponding *in vivo* assays to complement the *in vitro* cell line panels. Initially, it was thought that the *in vivo* follow-up of selectively active *in vitro* leads would be very time-consuming since the tumors would have to be established as subcutaneous implants in athymic mice, and growth of such xenografts to a size suitable for antitumor assays often takes several months. A new *in vivo* assay using microencapsulation technology has been developed, however, that looks very promising. Tumor cells harvested from actively growing cultures are trapped in small spherical capsules with semipermeable membranes, using technology developed by Damon Biotech Inc. These microcapsules are implanted into the peritoneal cavity of athymic mice where the tumor cells generally grow very well. Drugs can be administered by the intaperitoneal, subcutaneous, or intravenous route, and at the end of the experiment the capsules can be harvested and disrupted; tumor cells from control and treated animals can then be counted. The assay generally takes 5–10 days, making it suitable as a first stage *in vivo* model (Gorelik et al. 1986). By means of this system, the NCI expects that within the next 5 years, at least several interesting new agents with selectivity against slow-growing solid tumors will be identified as candidates for development towards clinical trials.

NATURAL PRODUCT ACQUISITION PROGRAM 1959–1985

Major NCI programs were established in 1959 to collect, identify, and screen plant products and fermentation broths. A modest marine acquisition program, established in 1972, focused almost entirely on animals. Through 1980 these programs generated and screened more than 200,000 fermentation broths and more than 100,000 plant extracts, but only 17,000 marine extracts (Douros and Suffness 1981). The plant and marine programs were deemphasized in 1981 as a result of minimal new discoveries of interest with mouse leukemia *in vivo* screens. The fermentation programs, which focused mainly on actinomycetes, were discontinued in 1986, when it was decided that focus should be on less investigated types of microorganisms (as described below).

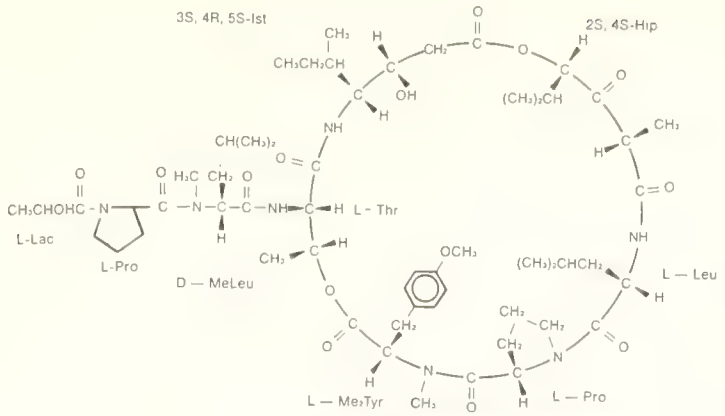

FIGURE 4. Molecular structure of didemnin B.

Activity rates of the marine animal extracts against the P388 mouse leukemia primary screen were comparable to those for the plant extracts and fermentation broths, with exceptional activity noticed in groups such as sponges and tunicates (Table 1). While high antitumor activity rates were encountered in fishes, the responsible substances were repeatedly found to be of high molecular weight (e.g., proteins) and thus not practical for development as cancer chemotherapeutics. It is still too early to ascertain the clinical activity of antitumor compounds isolated from these marine organisms, as none have yet completed clinical trials. Two, however, are of great interest to the NCI and will be discussed briefly below.

Didemnin-B is the first marine compound to enter cancer human clinical trials as the natural product. This cyclic peptide (Fig. 4) was isolated in 1981 from the Caribbean tunicate *Trididemnin solidum* independently by Dr. K. L. Rinehart's group at the University of Illinois (Rinehart et al. 1981) and by Dr. A. J. Weinheimer's group at the University of Houston. The compound is extremely potent against B16 murine melanoma and has broad activity against tumor stem cells in culture. The mechanism of action of didemnin-B is apparently through inhibition of protein synthesis (Crampton et al. 1984; Li et al. 1984). Substantial toxicity exhibited in dog studies was found to be dose-related and reversible, and thus the compound was approved as an Investigational New Drug in 1984 (Chun et al. 1986). Didemnin-B has now completed Phase I human clinical trials in which the maximum tolerated human dose was established. Phase II trials, to determine effectiveness of the drug against 21 human cancers, begins in summer 1988. The Phase II trials required recollection of 500 kg (net weight) of this thinly encrusting tunicate for bulk isolation of 200 g of the drug.

Another set of compounds of interest to the NCI and other groups for clinical development are the bryostatins, a group of macrocyclic polyether lactones isolated from the bryozoan *Bugula neritina* by Dr. G. R. Pettit's laboratory at Arizona State University (Pettit et al. 1982; Fig. 5). These compounds, which are extremely potent against P388 mouse leukemia and human ovarian sarcoma *in vivo*, act to modulate protein kinase C activity, a kinase that strongly influences proliferation of tumor cells (Smith et al. 1985). Further development of the bryostatins has been hampered by lack of sufficient supplies, but a large recollection of 11,000 gallons of *B. neritina* began in spring 1988.

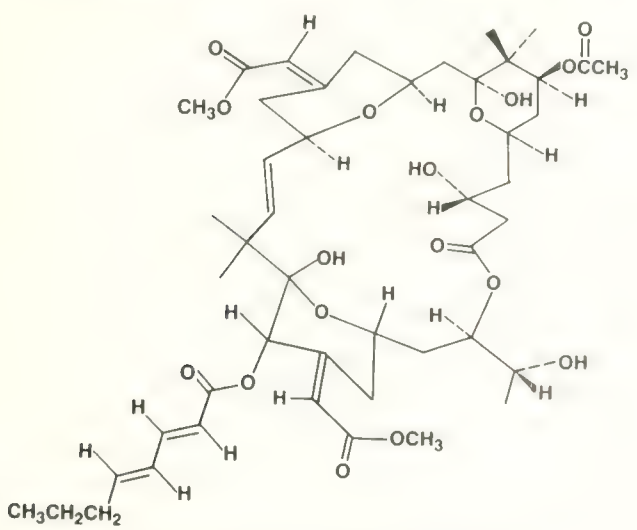

FIGURE 5. Molecular structure of bryostatin 1.

NEW NATURAL PRODUCT ACQUISITION PROGRAMS

New natural product acquisition programs were initiated in 1985–1986 in conjunction with the new *in vitro* human tumor panel described earlier. Contract collections proposed or already initiated are summarized in Table 2. Concentration is still on

TABLE 2. NUMBER AND SOURCES OF SAMPLES TO BE COLLECTED IN THE NCI PROGRAM FOR SCREENING DURING 1986–1991

Marine Macroorganisms	
	5,000, shallow water (< 30 m), Indo-Pacific temperate and tropical regions
	5,000, deep water (30–800 m), Caribbean and Atlantic regions
Tropical Rainforest Plants	
	7,500, Southeast Asia
	7,500, Africa and Madagascar
	7,500, Central and South America
Microorganisms	
	1,500, fungi—soil origin
	1,500, cyanobacteria—soil and other origin
	2,000, diverse marine microorganisms*

* Program to be initiated in 1988.

Table 3. Sample Quantities and Biological Data Required at Various Stages of Drug Development.

	Stages of drug development from natural sources		
	Initial collection	First recollection	Large-scale recollection
Purpose	Screening Fractionation	Isolation Chemical identification Pharmacology Formulation	Toxicology Clinical studies
Requirement	1 kg	50–200 kg	500–5,000 kg
Concurrent biological studies	Taxonomy Biology Ecology	Distribution Abundance Chemical variation	Physiological ecology Chemical ecology Alternate sources

the original three acquisitions areas (microbial, plant, and marine), but with much greater emphasis on marine macroorganisms, and on unusual microorganisms, including groups of marine origin. Two contracts were awarded to SeaPharm, Inc., for collection of 5,000 shallow-water macroorganisms by SCUBA diving and collection of 5,000 deep-water macroorganisms to depths of 830 meters using submersibles. Multiple contracts for isolation and fermentation of selected marine microorganism groups are to be awarded in 1988.

All the new acquisitions programs seek the greatest possible taxonomic and environmental diversity of organisms in order to provide the greatest possible diversity of chemical constituents. All materials will be extracted with aqueous and organic solvents, separately (by splitting the sample) or in series, to produce two extracts. A central feature of the new natural products program is a large repository for frozen storage of aliquots of these extracts to be used for any needed bioassay-directed chemical workup and for future screens.

SPECIAL CONSIDERATIONS FOR MARINE PRODUCT DEVELOPMENT

The stages of drug development from macroorganisms from an acquisitions point of view are illustrated in Table 3. Consideration of these stages is probably more critical for marine products than for terrestrial products as acquisition of bulk supplies is likely to be problematic for rare, poorly known, or deep-water organisms.

The primary sample documentation required by the NCI for its marine collections, noted under "Concurrent Biological Studies" in Table 3, includes extensive collection data, field notes on biology/ecology, photographic records, voucher specimens, and expert taxonomy. This level of sample documentation, which allows for recollection to obtain bulk supplies for development toward the clinic, is described in detail earlier in this volume by Dr. Shirley Pomponi, and will not be described further here.

When a biologically active extract is identified, measures will be taken to acquire additional biological information about the source organism to insure that sufficient supplies of the natural product will be available for preclinical and clinical development. Concurrent with the first recollection of 50–200 kg, as needed for further chemical workup and pharmacology studies (Table 3), biological studies will be initiated to establish the distribution and abundance of the source organism and to assess the extent of any variability in the compound(s) of interest within the organism's body and over the organism's geographical/bathymetric range. With later recollections for formulation, toxicology, and clinical studies, biological studies will focus on the physiological ecology of the source organism, with an emphasis on natural mechanisms that induce production of the compound(s). Considerations of sources of the compound(s) of interest other than by collection will also be made. They potentially include: 1) collection of close relatives; 2) microbial symbionts grown via fermentation; 3) aquaculture; 4) laboratory synthesis, semisynthesis, or microbial transformation of related compounds; 5) genetic engineering; and 6) tissue culture.

CONCLUSION

The marine environment remains relatively unexplored as a source of new anticancer drugs, but its potential is clear from the variety of biologically active novel structure types isolated in recent years. These observations, together with the introduction of a new *in vitro* human tumor screen, have prompted the NCI to promote the investigation of marine organisms as a major source of new compounds for the development of selective anticancer agents. Unique requirements for the development of drugs from marine organisms that are rare or difficult to collect are recognized. Thus the marine program includes provisions for studies of the distribution, abundance, and chemical ecology of such organisms producing promising compounds, as well as timely consideration of alternate sources of such compounds for the clinic.

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Concluding Remarks

The multidisciplinary nature of "Biomedical Importance of Marine Organisms"—the third biennial symposium of the California Academy of Sciences—provided an opportunity to assess the past and future of marine natural products research, of the use of marine organisms as model systems, and of evolutionary/phylogenetic studies derived from the preceding two. In comparing the past decade of research with contemporary studies, I was impressed with the spirit of optimism that has replaced disappointment, and with the sophistication this diverse field has achieved.

In the early days of marine natural products research—the late 1960s and early 1970s—studies were largely limited to simple compounds such as mono- and sesquiterpenoids, which were easy to isolate and describe structurally with the analytical methods then available. Over the past decade, developments in organic spectroscopy and separation science, particularly the innovations in nuclear magnetic resonance (NMR) and high-performance liquid chromatography (HPLC), have expanded the frontiers of natural products chemistry, as described, for example, by Ireland et al. Molecules of greater size and complexity, such as the polypeptides investigated by Kem, and Greenberg and Price, are now within reach of structural analysis. Innovations in the methods used to isolate and purify natural products have revealed that nature contains an array of sensitive molecules that were previously not observed because of harsh or otherwise inappropriate methods, as illustrated by Nakanishi. Hence, our view of naturally occurring compounds has expanded to include cyclic peroxides and hydroperoxides, cyclic ketals, and molecules that violate even the most well thought-out chemical principles such as Bredt's Rule. Using care and comprehensive methods has allowed natural products chemists access to an expanded realm of exciting drug candidates, and an even wider array of probes and clues to evolution, as evidenced by Faulkner.

Studies of marine organisms have expanded biologically, geographically, and bathymetrically. Whereas once only the most easily accessible intertidal and shallow water species, mainly from temperate climes, were available, modern technology has provided access to new resources. Deep-diving submersibles and remotely operated vehicles, described by Pomponi, now obtain samples of animals from depths that were virtually unexplored only a decade ago. Rinehart spoke of self-contained extraction and screening laboratories, made possible through advances in instrumentation, that can be taken onto ships and remote shores. In addition, since larger quantities of substances are needed than can sometimes be harvested, culture programs have been developed. And recent studies have revealed that marine microorganisms, such as those cultured by Moore et al. also produce complex, unprecedented compounds.

Chemists are collaborating with biologists in greater numbers than ever before. The nature of these associations appears to be changing. Early on—as many as 40 years ago—emphasis was on marine organisms as model systems, perhaps the most famous example of which involved neurophysiology of the squid, as described by Narahashi. Contemporary examples are at a much finer level of analysis—Taylor et al.'s investigations of toxins as probes of acetylcholine receptor structure, for example. Carr et al. illustrated that these may provide profound insights into evolution, but therein may also lie the explanation for the apparently reduced vigor of this facet of marine pharmacological research. Subtle differences between marine models and humans are increasingly detectable. While these may provide interesting pure scientific data, they diminish the potential for application in medicine.

Contemporary collaborations, which center increasingly on molecules rather than systems, yield data for chemosystematic (see Faulkner, and Greenberg and Price) and ecological (see Paul) studies. But they also to an increasing extent (and ethnographic leads to a minor degree, among them those described by Scheuer) have provided leads to promising organisms as sources of new compounds. Ascidians, for example, represent a group of invertebrates that only recently have become a focus of intense chemical study. Their systematics is complex, and little progress could be made on the natural products frontier without close collaboration between chemists and biologists. Winston pointed out that this cannot be a one-way street, however, and continued strengthening of cooperation will be possible only if both sides understand the needs and goals of the other.

The same is true of the interface between chemists and pharmacologists, where there is, at present, a strong imbalance toward chemical investigation. Despite the past decade's research having led to the overwhelming conclusion that marine resources are an exceptional reservoir of bioactive natural products, the field of marine pharmacology has been slow to develop. Of the thousands of newly isolated compounds for which structures have been published, fewer than 5% have been investigated by pharmacologists. This is more than unfortunate, since published structure types cannot be patented, and therefore are unlikely to be adopted for drug prototype development. Hence, the emphasis on chemistry has actually inhibited the development of marine compounds as pharmaceutical leads.

The future is bright, however, for marine pharmaceuticals, as described by Suffness and Thompson. Indeed, Wheeler et al. and Mayer and Jacobs presented work illustrating this frontier. Pharmacologists, like the biologists mentioned before, must be invited at an early stage to collaborate in order to guarantee that opportunities will not be lost.

In fact, the future is bright for this broad field we call marine pharmacology. Young academic chemists and pharmacologists are being attracted to this highly interdisciplinary area of study. As drug development has evolved into a demanding and precise science, marine organisms are providing exciting bioactivity leads, and structures of unprecedented classes are continually being isolated. The pharmaceutical industry, both in the United States and abroad, has begun to invest significant resources in marine chemistry and pharmacology (see, for example, Pomponi, and Rinehart). There seems no doubt that marine organisms will emerge within the next decade as a major resource for novel bioactive compounds for use both as research tools and as drugs. Growing knowledge of molecular biology means that model systems can serve increasingly specific purposes. Clearly, the biomedical importance of marine organisms can only be enhanced.

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